Volume 44 December 1958 Number 12

Proceedings of the National Academy of Sciences

of the United States of America

The Proceedings of the National Academy of Sciences

OFFICERS OF THE ACADEMY

DETLEV W. BRONK
President

FARRINGTON DANIELS
Vice President

H. P. ROBERTSON
Foreign Secretary

HUGH L. DRYDEN
Home Secretary

WILLIAM J. ROBBINS
Treasurer

EDITORIAL BOARD OF THE PROCEEDINGS

WENDELL M. STANLEY
Chairman

HUGH L. DRYDEN, *Home Secretary*
WILLIAM J. ROBBINS, *Treasurer*
H. P. ROBERTSON, *Foreign Secretary*
EDWIN B. WILSON, *Managing Editor*

GREGORY BREIT
HARRISON BROWN
ERNST CLOOS
THOMAS FRANCIS, JR.
C. H. GRAHAM
SAUNDERS MAC LANE
H. W. MAGOUN
HARLOW SHAPLEY
PAUL WEISS

Subscription price is $12.50 for 1 year, $24.00 for 2 years, and $34.50 for 3 years; the price of a single issue is $1.50. Make all remittances payable to the National Academy of Sciences in U.S. currency or its equivalent. Authorized agent for the British Commonwealth, except North America and Australasia: Cambridge University Press, Bentley House, 200 Euston Road, London, N. W. 1, England.

Editorial correspondence should be addressed to the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, 2101 Constitution Avenue, Washington 25, D. C.

Business correspondence should be addressed to the Printing and Publishing Office, National Academy of Sciences, 2101 Constitution Avenue, Washington 25, D.C.

Subscribers are requested to notify the Printing and Publishing Office of the Academy and their local postmaster immediately of change of address. Notices to the Academy should provide both the old and the new address.

Microfilms of complete volumes of this journal are available to regular subscribers only and may be obtained at the end of the volume year from University Microfilms, 313 N. First Street, Ann Arbor, Michigan.

Entered as second-class matter June 21, 1920 at the Post-office at Easton, Pennsylvania, under the Act of August 24, 1912. Accepted for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917. Authorised June 18, 1920.

PRINTED IN THE U. S. A.

THE PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
is published for
THE NATIONAL ACADEMY OF SCIENCES
by
THE UNIVERSITY OF CHICAGO PRESS

Proceedings of the

NATIONAL ACADEMY OF SCIENCES

Volume 44 · Number 12 · December 15, 1958

*PROTEIN BIOSYNTHESIS BY A CELL-FREE BACTERIAL SYSTEM. II. FURTHER STUDIES ON THE AMINO ACID INCORPORATION ENZYME**

By Mirko Beljanski† and Severo Ochoa

DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE, NEW YORK, NEW YORK

Communicated October 27, 1958

INTRODUCTION

The incorporation of amino acids into the protein of particulate preparations of *Alcaligenes faecalis* is stimulated by an enzyme present in the supernatant extract. This enzyme, which was isolated in highly purified form and is distinct from the known amino acid–activating enzymes catalyzing the amino acid–dependent exchange of PP^{32} with ATP,[1] appears to be concerned with some phase of protein biosynthesis.[2] The enzyme was found in extracts of *A. faecalis*, *Azotobacter vinelandii*, and *Escherichia coli*. Pending elucidation of its nature and mode of action, the new enzyme is referred to as the "amino acid incorporation enzyme."

Preliminary experiments, to be reported in this paper, indicate that the amino acid incorporation enzyme is involved in protein biosynthesis in mammalian as well as in bacterial cells. Thus (*a*) there are indications that the incorporation enzyme is present in liver extracts, and (*b*) highly purified preparations of the *A. faecalis* enzyme completely replace the liver supernatant or pH 5 enzymes of Hoagland *et al.*[3] in stimulating the incorporation of amino acids into protein of rat liver microsomes. We further wish to report that highly purified preparations of the *A. faecalis* incorporation enzyme catalyze a Mg^{++}-dependent exchange of radioactive ADP with ATP, a reaction which appears to be related to their amino acid incorporation activity. This finding may be of significance in view of the fact that glutathione synthetase, an enzyme involved in the synthesis of a typical peptide, catalyzes a similar exchange of ADP with ATP.[4]

MATERIALS AND METHODS

The preparation of *A. faecalis* particles, determination of the incorporation of C^{14}-labeled amino acids into protein, and purification of the amino acid incorporation enzyme have been previously described.[2] Microsomes, supernatant fluid, and pH 5 enzymes were prepared from the livers of young rats (80–100 gm.) as described by Keller and Zamecnik.[5] For measurement of the exchange of C^{14}-labeled ADP with ATP, samples were prepared and incubated as described in the legend to Table 3. The reaction was stopped by addition of 0.05 ml. of 50 per cent trichloroacetic

acid, and aliquots of the protein-free supernatant fluid were chromatographed in the isobutyric acid–ammonia system.[6] The ADP and ATP spots were located with an ultraviolet lamp, cut out, and eluted with 1.0 N HCl. The eluates were dried and their radioactivity measured with an end-window counter. The per cent exchange was calculated according to De Moss and Novelli.[7] 8-C^{14}-ADP was obtained from the Schwarz Laboratories, Mount Vernon, New York. GDP and crystalline ATP were purchased from the Sigma Chemical Company, St. Louis, Missouri. C^{14}-labeled amino acids were obtained from the New England Nuclear Corporation, Boston, Massachusetts. Phosphoenolpyruvate and crystalline pyruvic kinase were purchased from Böhringer and Sons, Mannheim, Germany.

RESULTS

Amino Acid Incorporation Enzyme in Rat Liver Supernatant.—As shown in the previous paper,[2] amino acid incorporation enzyme preparations, free of conventional amino acid–activating enzymes, stimulate the incorporation of C^{14}-labeled amino acids into the protein of washed *A. faecalis* particles. Table 1 shows that rat liver supernatant, the pH 5 enzymes derived from it, and the supernatant extract from *A. faecalis* cells produce the same degree of stimulation of incorporation of amino acids into protein of washed *A. faecalis* particles. These results strongly suggest that the liver fractions contain amino acid incorporation enzyme. However, as previously noted for the *A. faecalis* extract, part of the activity of the liver fractions is not destroyed by heat (Table 1) and is, presumably, non-enzymatic in nature.

TABLE 1

EFFECT OF RAT LIVER SUPERNATANT OR pH 5 ENZYMES ON INCORPORATION OF C^{14}-LABELED AMINO ACIDS INTO PROTEIN OF WASHED *A. fæcalis* PARTICLES*

PARTICLES	ADDITIONS	LABELED AMINO ACID INCORPORATED INTO PARTICLES (MILLIMICROMOLES PER MG. PROTEIN N OF PARTICLES PER HOUR) Glycine	D,L-Valine	D,L-Phenylalanine
Unwashed	None	26	..	..
Washed	None	13	5	21
	Heated liver supernatant (100 mg. protein)	17	..	..
	Liver supernatant (50 μg. protein)	19	..	..
	Liver supernatant (100 μg. protein)	21	10	32
	Liver supernatant (200 μg. protein)	22	..	..
	Alcaligenes supernatant (100 μg. protein)	23	..	..
	Heated pH 5 enzymes (100 μg. protein)	17	..	..
	pH 5 enzymes (50 μg. protein)	20	..	..
	pH 5 enzymes (100 μg. protein)	22	9	30

* The samples contained: Tris buffer, pH 7.0, 50 μmoles; $MgCl_2$, 5.0 μmoles; K_2HPO_4, 5.0 μmoles; C^{14}-labeled amino acid (specific radioactivity, 80,000–124,000 cpm per μmole), 0.25 μmole; and *A. fæcalis* particles with 300 μg. of protein. Rat liver supernatant, pH 5 enzymes, or *A. fæcalis* supernatant added as indicated. Final volume, 1.0 ml. Incubation in air, 1 hour at 32°.

Effect of Incorporation Enzyme on Amino Acid Incorporation by Rat Liver Microsomes.—Table 2 shows, in agreement with the results of previous workers, that liver pH 5 enzymes strongly stimulate the incorporation of C^{14}-leucine into the protein of rat liver microsomes. This effect has been ascribed to the amino acid–activating enzymes present in this preparation. However, as further shown in Table 2, small amounts (10 μg. of protein) of purified *A. faecalis* incorporation enzyme, free of amino acid–activating enzymes, were as effective as the pH 5 enzymes (1.5 mg. of protein) in stimulating the incorporation of leucine into microsomal protein. Contrary to the *A. faecalis* particles, in which the ATP required for amino acid

incorporation is generated by oxidative phosphorylation,[2] the microsome system functions anaerobically, ATP being provided as such and regenerated from ADP by transfer of phosphate from phosphoenolpyruvate in the presence of pyruvic kinase. Therefore, the results of Table 2 reinforce previous evidence[2] that the action of the amino acid incorporation enzyme is not due to an effect on oxidative phosphorylation.

TABLE 2

EFFECT OF AMINO ACID INCORPORATION ENZYME ON INCORPORATION OF C^{14}-LEUCINE INTO PROTEIN OF RAT LIVER MICROSOMES*

Microsomes		C^{14}-Leucine Incorporated (cpm per mg. Microsomal Protein)
Unwashed	None	20
	Heated pH 5 enzymes	20
	Heated incorporation enzyme	21
	pH 5 enzymes	95
	Incorporation enzyme	98
Washed†	None	12
	Heated pH 5 enzymes	13
	Heated incorporation enzyme	11
	pH 5 enzymes	58
	Incorporation enzyme	59

* The samples contained: D,L-C^{14}-leucine, 0.2 μmole (144,000 cpm); ATP, 1.0 μmole; GDP, 0.25 μmole; phosphoenolpyruvate, 10 μmoles; pyruvic kinase, 50 μg.; and microsomal suspension (in a solution [Keller and Zamecnik, *J. Biol., Chem.*, **221** 45, 1956] containing 0.35 *M* sucrose, 0.035 *M* $KHCO_3$, 0.025 *M* KCl, and 0.004 *M* $MgCl_2$) with 4.0 mg. of protein. Other additions: pH 5 enzymes with 1.5 mg. of protein; *A. fæcalis* amino acid incorporation enzyme, specific activity (glycine) 24[2], with 10 μg. of protein. Final volume 1.0 ml. Incubation in 95 per cent N_2–5 per cent CO_2, 20 minutes at 37°.

† Washed once with suspending solution.

ADP-ATP Exchange Reaction with Incorporation Enzyme.—Highly purified preparations of the amino acid incorporation enzyme have been found to catalyze a rapid exchange of C^{14}-ADP with ATP which is strictly dependent on the presence of Mg^{++} (Table 3). There is no change in the concentration of either nucleotide. The rate of this reaction is not influenced by the addition of a mixture of amino acids (total concentration 2×10^{-5} to 2×10^{-3} *M*) and there is no inhibition by 2,4-dinitrophenol (up to 2×10^{-4} *M*) or chloramphenicol (up to 0.1 mg. ml.). The enzyme preparations were free of polynucleotide phosphorylase, inorganic pyrophosphatase, and adenylic kinase (myokinase). No exchange of P^{32} or PP^{32} with ATP was obtained whether in the absence or presence of amino acids. The virtual absence of adenylic kinase (which catalyzes the reaction 2 ADP = ATP + AMP) was established through (*a*) the absence of formation of labeled AMP and ATP on incubation of the incorporation enzyme with C^{14}-ADP, in the presence of Mg^{++}, and (*b*) the lack of oxidation of DPNH on incubation of the enzyme with ATP, AMP, Mg^{++}, phosphoenolpyruvate, pyruvic kinase, and lactic dehydrogenase. Had adenylic kinase been present, phosphoenolpyruvate would have transferred P to ADP (formed from ATP + AMP), and the resulting pyruvate would have been reduced to lactate by DPNH. Exclusion of adenylic kinase is important because the action of this enzyme would lead to ADP-ATP exchange. Although the absence of other enzymes, such as nucleoside monophosphokinases other than adenylic kinase and nucleoside diphosphokinases, has not yet been excluded, there is a fact that strongly suggests a relationship between the stimulation of amino acid incorporation and the ADP-ATP exchange. As previously reported,[2] highly purified preparations of the incorporation enzyme are very unstable and lose most of their activity in about 2 weeks when stored at −18°. We find that the amino acid

incorporation and the ADP-ATP exchange activity of these preparations both disappear at about the same rate. If the exchange is indeed related to the amino acid incorporation activity, the enzyme might be concerned with some kind of activation reaction, since a Mg^{++}-dependent ADP-ATP exchange is also brought about, in the absence of any other reactants, by the phosphorylating enzyme[8] and, as already mentioned, by glutathione synthetase.[4] The former enzyme catalyzes the reaction of succinate with ATP and CoA to succinyl CoA, ADP, and P, while the latter catalyzes the reaction of γ-glutamylcysteine, glycine, and ATP to glutathione, ADP, and P. Glutathione synthetase, kindly provided by Dr. Konrad Bloch, Harvard University, failed to replace the amino acid incorporation enzyme for the incorporation of glycine, valine, or phenylalanine, by washed *A. faecalis* particles.

TABLE 3

EXCHANGE OF C^{14}-ADP WITH ATP*

EXPERIMENT No.	SYSTEM	INCUBATION TIME (MINUTES)	RADIOACTIVITY (cpm) ADP	RADIOACTIVITY (cpm) ATP	EXCHANGE Per Cent	EXCHANGE μMoles/mg. Protein/Hour
1	Complete	0	3,600	60	0	0
	Complete	5	2,900	720	39	594
	Complete	10	2,389	1,206	64	610
2	Complete	0	4,330	72	0	0
	Complete	5	3,520	830	37	552
	Complete	10	3,000	1,331	59	536
	No $MgCl_2$	10	4,350	62	0	0
	No enzyme	10	4,340	70	0	0
	No ATP	10	4,346	65	0	0

* The complete system contained: Tris buffer, pH 7.0, 50 μmoles; $MgCl_2$, 1.0 μmole; C^{14}-ADP, 1.0 μmole (4,500–9,000 cpm); crystalline ATP, 1.0 μmole; and freshly prepared *A. faecalis* amino acid incorporation enzyme, specific activity (glycine) 24 (experiment 1) or 21 (experiment 2), with 5 μg. of protein. Final volume, 0.5 ml. Incubation in air at 34°.

SUMMARY

1. Indications have been obtained for the presence of the amino acid incorporation enzyme in the supernatant fraction from rat liver and in the precipitate obtained by acidification of this fraction to pH 5.2 (pH 5 enzymes). Highly purified amino acid incorporation enzyme from *A. faecalis* completely replaces the pH 5 enzymes in stimulating the incorporation of C^{14}-leucine into protein of rat liver microsomes. These observations show that the amino acid incorporation enzyme is involved in protein biosynthesis in both mammalian and bacterial cells.

2. Highly purified preparations of the *A. faecalis* incorporation enzyme catalyze a rapid, Mg^{++}-dependent exchange of radioactive ADP with ATP an activity which appears to be related to their amino acid incorporation activity. This finding may be of significance, since glutathione synthetase, which catalyzes the synthesis of a typical peptide, brings about a similar exchange.

* Aided by grants from the National Institute of Arthritis and Metabolic Diseases (Grant A-1845) of the National Institutes of Health, United States Public Health Service, and the Rockefeller Foundation.

† Present address: Service de Biochimie Cellulaire, Institut Pasteur, 28 rue du Docteur Roux, Paris XV, France.

[1] Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine-5′-monophosphate; P, orthophosphate; PP, pyrophosphate; DPNH, reduced diphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane; cpm, counts per minute.

[2] M. Beljanski and S. Ochoa, these PROCEEDINGS, **44**, 494, 1958.
[3] M. B. Hoagland, E. B. Keller, and P. C. Zamecnik, *J. Biol. Chem.*, **218**, 345, 1956.
[4] J. E. Snoke and K. Bloch, *J. Biol. Chem.*, **213**, 825, 1955.
[5] E. B. Keller and P. C. Zamecnik, *J. Biol. Chem.*, **221**, 45, 1956.
[6] H. A. Krebs and R. Hems, *Biochim. et Biophys. Acta*, **12**, 172, 1953.
[7] J. A. De Moss and G. D. Novelli, *Biochim. et Biophys. Acta*, **22**, 49, 1956.
[8] S. Kaufman, *J. Biol. Chem.*, **216**, 153, 1955.

---

*ON THE SEPARATION OF THE TRYPTOPHAN SYNTHETASE OF ESCHERICHIA COLI INTO TWO PROTEIN COMPONENTS**

BY IRVING P. CRAWFORD† AND CHARLES YANOFSKY

DEPARTMENT OF BIOLOGICAL SCIENCES, STANFORD UNIVERSITY, STANFORD, CALIFORNIA

Communicated by E. L. Tatum, October 6, 1958

INTRODUCTION

Tryptophan synthetase (TSase), the enzyme catalyzing reaction (1),

$$\text{Indole} + \text{L-serine} \rightarrow \text{L-tryptophan}, \qquad (1)$$

has been demonstrated in a variety of micro-organisms.[1] Recent studies with partially purified TSase preparations from *Neurospora crassa* have shown that this enzyme also acts on indoleglycerol phosphate (InGP), a precursor of tryptophan, according to reactions (2) and (3).[2]

$$\text{Indoleglycerol phosphate} \rightleftharpoons \text{indole} + \text{triose phosphate}, \qquad (2)$$

$$\text{Indoleglycerol phosphate} + \text{L-serine} \rightarrow \text{L-tryptophan} + \text{triose phosphate}. \qquad (3)$$

These studies further indicated that reaction (3) rather than reactions (1) plus (2) probably represents the principal physiological mechanism of tryptophan synthesis in *N. crassa*, since evidence was obtained excluding free indole as an intermediate in this reaction.

Investigations with *Escherichia coli* have shown that two proteins, designated components A and B, are required for the catalysis of reaction (2) and that one of these, component B, is also involved in reaction (1).[3] In view of these findings and the recent observations with preparations from *N. crassa*, a study of reactions (1), (2), and (3) in extracts of *E. coli* was undertaken. It was soon found that the same two separable protein components were required for the catalysis of all three reactions. This paper is concerned with the demonstration of the separability of these two proteins and with certain unusual features of their interaction.

MATERIALS AND METHODS

Organisms.—Two tryptophan auxotrophs of *E. coli* K-12 were used as enzyme sources, strains T-3 and T-8. Mutant T-3 is blocked in the synthesis of anthranilic acid and forms large amounts of TSase when grown on low levels of anthranilic acid.[4] It was considered equivalent to wild-type for the purposes of this study. Mutant T-8 responds to indole or tryptophan but not to anthranilic acid and

accumulates indoleglycerol in its culture filtrates.[4] Extracts of this strain cannot convert InGP to indole or to tryptophan.

Enzyme Assays.—Reaction 1 (indole + serine → tryptophan) was measured as described previously.[3] Indole disappearance was determined from a reaction mixture containing indole, serine, pyridoxal phosphate, sodium chloride, and tris buffer at pH 7.8. Indole disappearance under these conditions was accompanied by the formation of equimolar amounts of tryptophan. One unit of activity corresponds to the conversion of 0.1 μ M of indole to tryptophan in 20 minutes at 37° C. under standard assay conditions. Specific activity is expressed as units of activity per milligram protein. Protein was determined by the method of Lowry *et al.*[5]

The conversion of InGP to tryptophan was carried out in a reaction mixture identical with the above except that InGP was substituted for indole. The extent of the reaction was determined by measuring the tryptophan content of a perchloric acid deproteinized sample of the incubation mixture. Tryptophan was determined microbiologically using strain td_4, an auxotroph of *E. coli* K-12 which responds to L-tryptophan specifically.[6] One unit of activity corresponds to the conversion of 0.1 μ M of InGP to tryptophan in 20 minutes at 37° C. under standard assay conditions.

Since, as will be shown presently, two protein components, A and B, are involved in the reactions described above, procedures were devised for quantitatively measuring each component. Component A assays were performed as described above with the addition of a three-fold or greater excess of component B, and component B assays were performed in the presence of a three-fold or greater excess of component A. The activity detected in either case was expressed as units of component A (assayed with excess B) or units of component B (assayed with excess A), where the unit again refers to the conversion of 0.1 μ M of substrate to product in 20 minutes at 37° C. Partially purified component A, free of component B activity, was used for the assay of component B, while T-8 preparations served as a source of component B for the assay of component A. A small correction was necessary in component A assays when indole → tryptophan activity was measured, since T-8 preparations exhibit a slight amount of indole → tryptophan activity in the absence of component A. This activity is increased twenty to thirty fold, however, in the presence of component A.

Partial Purification of TSase.—All operations were performed at 0–6° C. Extracts of *E. coli* T-3 were prepared by disrupting lyophilized cells suspended in 0.1 M potassium phosphate buffer, pH 7.8, with No. 130 Superbrite glass beads in a Nossal vibrator. After centrifugation for 30 minutes at 20,000 × g. to remove debris, these extracts were heated at 58° C. for 3 minutes and the denatured protein removed by centrifugation. Nucleic acids were precipitated by the addition of one-half volume of 20 per cent streptomycin sulfate. The supernatant solution was then brought to pH 6.2–6.3 by the addition of 1 M sodium acetate buffer, pH 4.5. Proteins precipitating from this solution between 33 and 43 per cent saturation with ammonium sulfate were collected by centrifugation and redissolved in column buffer. The column buffer was 0.01 M potassium phosphate at pH 7.0, generally supplemented with pyridoxal phosphate and reduced glutathione at final concentrations of 2×10^{-5} M and $10^{-4}M$, respectively. The

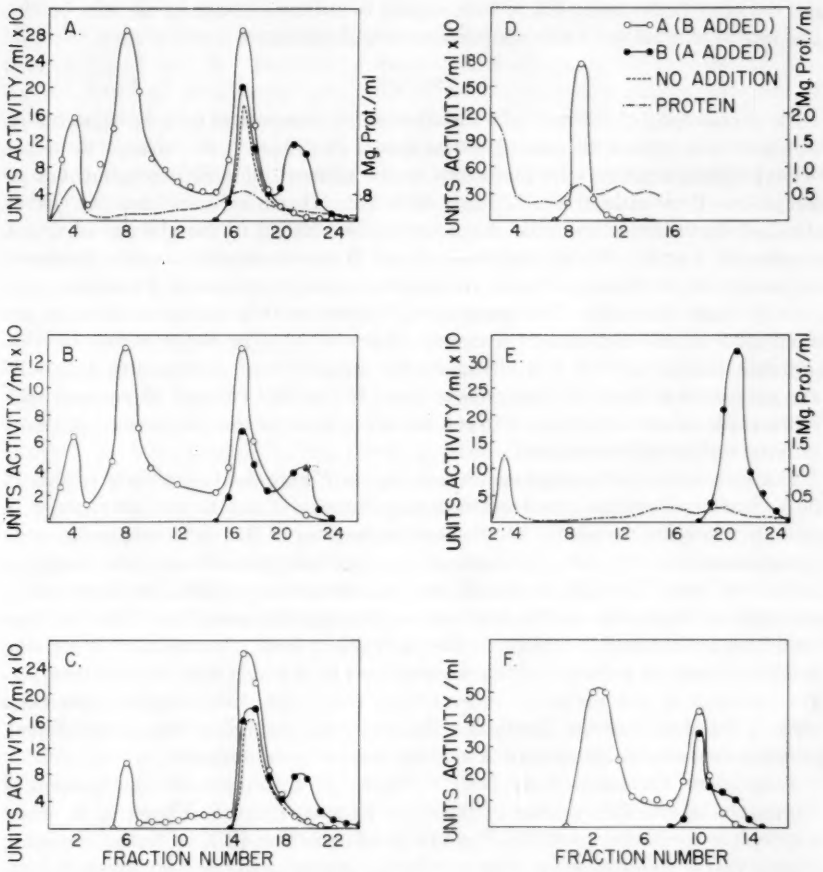


FIG. 1.—Chromatography of partially purified *E. coli* preparations on diethylaminoethyl cellulose. (*A*) T-3 TSase assayed by indole → tryptophan. (*B*) T-3 TSase assayed by InGP → tryptophan. (*C*) Rechromatography of center (combined) peak fractions. (*D*) Partially purified T-3 component A. (*E*) Partially purified T-8 component B. (*F*) Mixture of chromatographically purified T-3 component A and T-8 component B. *C–F* assayed by indole → tryptophan. For additional explanation see text.

enzyme preparation was dialyzed for 1–2 hours against 50 volumes of column buffer. The next step in the purification procedure involved chromatography on diethylaminoethyl cellulose.[7, 8] The diethylaminoethyl cellulose employed was washed and suspended in 0.01 *M* potassium phosphate buffer at pH 7. When formed into a column of the desired diameter and at least 20 cm. in height, it was washed with about 2 volumes of column buffer. The dialyzed enzyme sample was then added to the column and washed on with one-half column volume of buffer. The amount of protein placed on a column never exceeded 2 mg./cc. of cellulose. Elution was accomplished by a gradient system employing approximately five times the column volume of supplemented buffer in the mixing bottle

and the same buffer made 0.5 *M* with respect to sodium chloride in the inlet bottle. The rate of elution was 1 column volume every 2–10 hours.

RESULTS

Chromatography of TSase.—The distribution of protein and enzymatic activities obtained in a typical chromatogram is shown in Figure 1 *A*. Assays of indole → tryptophan activity were performed in the presence of added component A or component B or without the addition of either. It can be seen from the curves obtained that there is one peak of activity (tubes 16–20) in the absence of added component A or B. When components A and B were assayed separately, however, component B exhibited a bimodal distribution, while three peaks of component A activity were observed. The recovery of indole → tryptophan activity in the fractions from this experiment was only 52 per cent. The major reason for this apparent loss of activity was obviously the separation of components A and B. The recoveries of these two components were 96 per cent (A) and 80 per cent (B). When these values were taken into consideration, most of the indole → tryptophan activity was actually recovered.

The distribution of component A activity in Figure 1, *A*, is clearly triphasic. In a number of similar experiments the appearance of the first small peak of A activity was quite variable. More constant features of the chromatography were the appearance of the bulk of component A at the relative position of the second or largest "A" peak (around fraction 8) and the appearance of less, but appreciable, amounts of A activity in the fractions separating this peak from the fractions exhibiting both A and B activity. The early minor peak of component A activity parallels a peak of protein-reacting material, some of which may be streptomycin. The behavior of component A under certain other conditions suggests that both early A fractions may be identical. However, the possibility that two different proteins may exhibit component A activity has not been excluded.

Tryptophan Formation from InGP.—Figure 1, *B*, shows the distribution of component A and B activities in the same chromatogram as Figure 1, *A*, when tryptophan formation from InGP is measured (reaction [3]). Clearly, the same protein pair is responsible for both reactions. Assays performed on fractions from a similar chromatogram indicate that the separation of the components required for the conversion of InGP to indole also follows the same pattern as that observed when reactions (1) and (3) are measured. It appears from these findings that all three reactions are catalyzed by the same pair of proteins.

Rechromatography of TSase-containing Fractions.—The appearance of both separate and combined peaks of components A and B on diethylaminoethyl cellulose chromatograms suggested that there was an association of the two proteins under the elution conditions employed. To examine this association further, fractions comprising the center or combined peak of the TSase chromatogram were concentrated by ammonium sulfate precipitation and rechromatographed under standard conditions. The distribution of activities obtained is shown in Figure 1, *C*. It is clear that appreciable amounts of free A and B separated from the combined peak fractions; thus the essential characteristics of the first chromatogram were duplicated, suggesting that the center peak represents an AB association.

Chromatography of a Mixture of Purified Components A and B.—As a further

test of AB association, a chromatogram was performed with a mixture of purified components A and B. For this purpose, modifications of the purification procedure described previously were utilized which yielded workable amounts of chromatographically homogeneous A and B. With component A the modification consisted of substituting a pH precipitation step for the ammonium sulfate precipitation in the purification procedure detailed above. Lowering the pH to 3.9 by the addition of 1 *M* sodium acetate buffer, pH 3.0, resulted in the precipitation of component B along with other contaminating proteins. After thorough dialysis, chromatography of the supernatant solution yielded the distribution of proteins and component A activity shown in Figure 1, *D*. Extracts of the mutant T-8 were used as a source of component B, since, as mentioned previously, T-8 preparations exhibit little or no A activity under the assay conditions employed. The purification procedure paralleled that used for T-3 TSase except for the ammonium sulfate step. With T-8 preparations those proteins precipitating between 0 and 33 per cent of saturation were carried on to constitute the column charge. A chromatogram of this partially purified component B is illustrated in Figure 1, *E*. When fractions containing chromatographically homogeneous A and B so obtained were combined and used as a column charge, the distribution of activities shown in Figure 1, *F*, was obtained. Even though an excess of component A was present, the similarity to Figure 1, *A* and *C*, is readily apparent. It is possible to obtain greater separation of components A and B from combined peak fractions and from mixtures if the protein solution is diluted before addition to the column and if the elution rate is decreased. However, conditions have not yet been found which give complete separation of A and B on diethylaminoethyl cellulose columns.

Recovery of A from Fractions Containing Excess B.—Although all the early chromatographic fractions rich in component A are incapable of converting indole to tryptophan, all the purified component B fractions obtained to date exhibit low levels of this activity. The level of activity observed with the best chromatographically purified T-3 and T-8 component B fractions is generally 3–5 per cent of that obtainable on the addition of component A. Experiments involving pH 4.0 precipitation were performed with such preparations in an attempt to determine whether or not the low level of activity detected was due to small amounts of contaminating component A. At least half the "inherent" indole to tryptophan activity of a chromatographically purified T-3 component B fraction could be accounted for in this manner, while no acid-stable material exhibiting component A activity could be recovered from T-8 component B fractions. Further studies are required before it can be established whether or not contaminating component A (probably an altered A in the case of T-8, since T-8 fractions show no InGP → tryptophan activity) is responsible for the low level of indole → tryptophan activity observed.

Properties of Purified Components A and B.—In its response to physical agents,[2] its susceptibility to destruction by trypsin, and its ability to elicit the production of neutralizing and precipitating antibodies,[9] component B exhibits the properties of a typical protein. Chromatographically purified component A is also protein-like in behavior, undergoing denaturation at temperatures above 55° C. and at extremely high and low pH values. In addition, incubation with either trypsin or pepsin results in the rapid inactivation of component A.

Further purification of T-3 component A has yielded a preparation with a specific activity of about 4,000, which migrates as a single component in the ultracentrifuge.[10] This specific activity represents a value approximately 1,000 times that obtained with crude extracts of strain K-12. The most highly purified component B prepared to date has a specific activity of 1,000, or 400 times greater than the value obtained with crude extracts of strain K-12.

Further Experiments on the Reaction between Proteins A and B.—In assaying combined peak chromatogram fractions (e. g., *17* and *18*, Fig. 1, *A*) containing equivalent amounts (activity) of the two components, it became apparent that neither component was fully active. Only when an excess of one component was added, was an accurate estimate of the other obtained. This relationship was examined further in a saturation experiment employing varying amounts of component A (Fig. 2). The results obtained show that approximately a three-fold

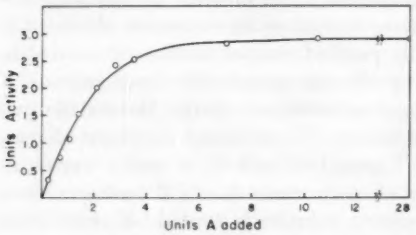


FIG. 2.—Saturation of component B with component A. Reaction mixtures (1-ml. volume) contained 0.4 μM indole, 4×10^{-5} M pyridoxal phosphate, 6×10^{-2} M DL-serine, 0.5 mg. bovine albumin, 10 mg. NaCl, 2.9 units of chromatographically purified T-8 component B, and varying amounts of chromatographically purified T-3 component A.

excess of component A is required for B to be maximally active. In other studies of AB interaction the rate at which these two proteins combine and interact was found to be quite rapid. On mixing the two components at 37° C., the lag observed prior to attaining the maximal rate of indole to tryptophan conversion was, at most, about 2 minutes. Dilution of the enzyme components in the presence of constant substrate concentrations did not bring about a detectable diminution in the reaction rate, though experimental difficulties limited this experiment to a tenfold dilution, and the reliability of the assay procedures at these levels probably was not better than 10 per cent.

Experiments Excluding a Small Molecular-Weight Intermediate.—With two protein components required for the catalysis of several rather complex reactions, even though a physical combination of the proteins appears to occur, it becomes necessary to search assiduously for a free intermediate compound. Two types of experiments were performed in the search for such a compound. The first approach, similar to that employed in studies with *N. crassa*,[2] tested the possible participation of free indole as an intermediate in reaction (3). In this experiment an extract of a mutant (td_2) of *E. coli* was used which was unable to convert indole or InGP to tryptophan but could hydrolyze InGP to indole. When mixed with an extract of a *N. crassa* mutant (A 78) possessing the ability to form tryptophan from indole but unable to act on InGP at an appreciable rate (Rachmeler, unpublished), tryptophan formation from InGP at a rate equal to that of the T-3 preparation would be expected if free indole were an intermediate in reaction (3). As can be seen from the data in Table 1, however, the amount of tryptophan formed by the A 78-td_2 mixture was insignificant compared with the amount formed by the T-3 extract.

Thus in *E. coli* as in *N. crassa*, free indole is not an intermediate in reaction (3).

The second experimental approach employed in the search for an intermediate involved measuring the various reactions when the two protein components were separated by a dialysis membrane (Table 2). Experiments 1 and 2 show that InGP is not converted to indole or to tryptophan when components A and B are so separated. Similarly, in experiment 3, indole disappearance was not significantly greater when the two components were separated than when each was present alone, while appreciable amounts disappeared when the two components were on the same side of the membrane.

Pyridoxal Phosphate Dependence.—Pyridoxal phosphate is required for the conversion of indole to tryptophan in both *Neurospora*[11] and *E. coli*. The pyridoxal phosphate dependence of reactions (2) and (3) was also investigated with *E. coli* preparations, and it was found that, of the three reactions catalyzed by this enzyme system, only those involving serine (reactions [1] and [3]) require pyridoxal phosphate. Pyridoxal phosphate also serves to stabilize both *Neurospora* TSase[12]

TABLE 1

EXCLUSION OF FREE INDOLE AS INTERMEDIATE IN CONVERSION OF INGP TO TRYPTOPHAN IN *E. coli*

EXTRACTS TESTED	ACTIVITY DETECTED: InGP → indole (μM/30 min)	Indole → tryptophan (μM/30 min)	InGP → tryptophan (μM/30 min)
T-3 0.1 ml.	0.03	0.57	0.3
td_2* 0.02 ml.	0.045	0	0
A78† 0.02 ml.	0	0.6	0.01
A78 0.02 ml. + td_2 0.02 ml.	....	...	0.04
T-3 0.1 ml. + td_2 0.02 ml.	....	...	0.29

* td_2 is a mutant of *E. coli* with an altered component B. Extracts of this strain will convert InGP → indole but will not convert InGP → tryptophan or indole → tryptophan.

† A78 is a tryptophan auxotroph of *N. crassa*. Partially purified TSase preparations from this organism exhibit indole → tryptophan activity but convert InGP → indole and InGP → tryptophan very poorly as compared with TSase preparations from wild-type *N. crassa* (Rachmeler, unpublished).

TABLE 2

EXCLUSION OF DIALYZABLE INTERMEDIATES

EXPERIMENT*	INSIDE DIALYSIS BAG: Enzyme	Substrate	OUTSIDE DIALYSIS BAG: Enzyme	Substrate	ACTIVITY: μM Indole	μM Tryptophan
1	A	InGP	B	InGP	...	0
	...	InGP	A + B	InGP	...	+1.59
2	B	InGP	A	InGP	0	...
	...	InGP	A + B	InGP	+0.15	...
3	A	Indole	B	Indole	−0.35	...
	A	Indole	...	Indole	−0.03	...
	...	Indole	B	Indole	−0.29	...
	...	Indole	A + B	Indole	−1.46	...

* Experiment 1: Each 4-ml. reaction mixture (1-ml. inside 1/4-inch dialysis bag, 3 ml. outside) contained 1.6 μM InGP, 2.4×10^{-2} *M* serine, 4×10^{-5} *M* pyridoxal phosphate, 9×10^{-2} *M* tris buffer pH 7.8, 1.7×10^{-1} *M* NaCl, partially purified T-3 component A and T-8 component B distributed as indicated.

Experiment 2: Each 2-ml. reaction mixture (0.5 ml. inside 1/4-inch dialysis bag, 1.5 ml. outside) contained 0.3 μM InGP, 0.1 *M* KPO_4 buffer pH 7.0, 7.5×10^{-3} *M* NH_4OH, partially purified T-3 component A and T-8 component B distributed as indicated.

Experiment 3: Reaction mixtures identical to experiment 1 except for the substitution of 1.6 μM of indole for InGP. Partially purified T-3 components A and B distributed as indicated.

and *E. coli* TSase. It was readily determined that this stabilizing effect was exerted on protein B specifically.

Experiments with P^{32}-Triose Phosphate.—If, as proposed on the basis of studies with *N. crassa*,[2] enzyme-bound indole serves as an intermediate in reaction (3), the glycerol phosphate moiety of InGP might be expected to exchange with triose phosphate. In a series of experiments employing P^{32}-triose phosphate and C^{14}-

indole, this possibility was examined (Table 3). The results obtained indicate that no such exchange occurs (experiment 1, tube No. 4). Furthermore, although indole and triose phosphate were readily converted to InGP, the presence of even small amounts of InGP in the reaction mixture suppressed the appearance of P^{32} and C^{14} in the isolated InGP. Since the incorporation of both indole and triose phosphate was inhibited to approximately the same extent (experiments 1 and 3), it appears that incorporation of either of these compounds into InGP is associated with the synthesis of InGP.

TABLE 3*

INCORPORATION OF P^{32}-TRIOSE PHOSPHATE AND C^{14}-INDOLE INTO INGP

EXPERIMENT	ENZYMES ADDED	UNITS ADDED	SUPPLEMENTS InGP (μM)	SUPPLEMENTS Indole (μM)	OBSERVED CPM IN INGP	PER CENT INHIBITION
1	A + B	28A + 11B	....	1	11,700	
1	A + B	28A + 11B	0.3	1	3,250	74
1	A + B	28A + 11B	0.6	1	1,140	91
1	A + B	28A + 11B	0.6	....	155	
1 control	A + B	28A + 11B	....	....	170	
2	A + B	30B + 1.8A	....	1	2,027	
2	A	200A	....	1	894	
2	A	200A	0.3	1	248	79
2	B	60B	....	1	58	
2 control	...	...	....	1	72	
3	A + B	28A + 11B	....	1	80	
3	A + B	28A + 11B	0.3	1	36	69
3	A + B	28A + 11B	0.6	1	28	81
3 control	...	...	....	1	16	

* All experimental tubes contained phosphate buffer at pH 7.0, 3 μM of hexosediphosphate and crystalline aldolase. In experiments 1 and 2 the hexosediphosphate was labeled (P^{32}), while in experiment 3 the indole was labeled (C^{14}). After a 30-minute incubation period the InGP formed was adsorbed on charcoal, the charcoal washed repeatedly, plated, and counted. In the experiment with C^{14}-indole, the residual indole was extracted with toluene, and the incubation mixture was treated with periodate to convert InGP to indole aldehyde. The indole aldehyde was extracted with ethyl acetate, and an aliquot of the extract was evaporated and counted. Labeled hexosediphosphate was prepared using P^{32} inorganic phosphate, and was purified by chromatography on Dowex-1 ion-exchange resin. Triose phosphate was generated from hexosediphosphate during the course of the experiment with aldolase. C^{14} indole was isolated from the culture filtrates of an indole-accumulating mutant of *E. coli* grown on C^{14}-acetate.

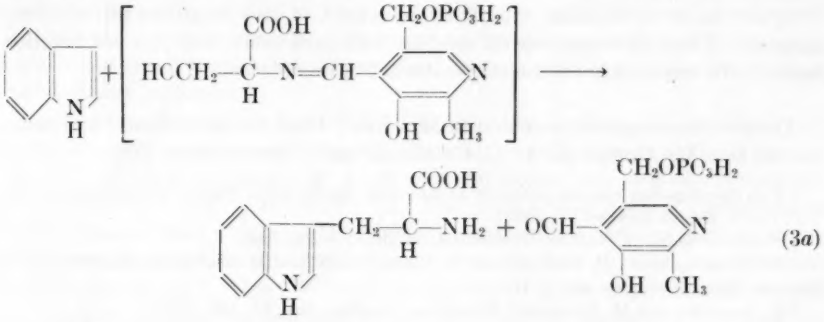
Examination of our most highly purified component A preparations revealed that they do incorporate P^{32}-triose phosphate into a charcoal-adsorbable compound in the presence of indole (experiment 2). As can also be seen in experiment 2, per unit of component A activity this conversion is only 1 per cent of that obtained in the presence of component B. This incorporation appears to represent InGP synthesis, since the reaction was indole-dependent and was strongly inhibited by InGP. T-8 component B exhibited no activity in the absence of added component A.

DISCUSSION

The chromatography of proteins is becoming an increasingly powerful tool in the study of enzymes. To our knowledge, however, it has not previously uncovered an enzymatic relationship analogous to that shown by the two proteins catalyzing the final step in tryptophan biosynthesis in *E. coli*. The evidence presented in this paper clearly indicates that these two proteins must undergo physical contact prior to attaining full activity, forming thereby a complex with new catalytic, as well as chromatographic, properties.[13] It seems unlikely that free intermediates are involved in any of the three reactions catalyzed by this

protein pair, and it appears probable that all three reactions are catalyzed on a single surface made active by the joining of components A and B. The name "tryptophan synthetase" will be retained for the A-B association catalyzing reactions (1), (2), and (3). It is of interest that the genes controlling proteins A and B are very closely linked on the bacterial chromosome and may in fact be adjacent to one another (C. Yanofsky and E. Lennox, unpublished).

Mechanistically viewed, the three reactions are not dissimilar. Reaction (3) may be considered analogous to reaction (2), the hydrolysis of InGP, except that a pyridoxal phosphate–serine compound of the type formulated by Braunstein and others [14–17] substitutes for water (reaction [3*a*]):



(3*a*)

In the same manner reaction (1) may be considered analogous to the reverse of reaction (2), with the pyridoxylidine azomethine compound (in brackets) substituting for triose phosphate. It should be noted that reaction (2), in either direction, is much slower than the reactions involving pyridoxal phosphate and serine; it proceeds at less than 5 per cent the rate of reactions (1) and (3).

The hypothetical pyridoxylidine azomethine compound shown has been assumed to be stabilized in an active form by metal chelation[16] or by attachment to a protein molecule.[18] The involvement of this hypothetical compound with its many tautomeric forms might explain the finding of Tatum and Shemin[19] that the α-hydrogen of serine is probably removed during the reaction between indole and serine.

The available data do not permit any definite conclusions on the function of each of the protein components of TSase. The finding that trace amounts of InGP → indole activity are associated with purified component A and that component B is stabilized by pyridoxal phosphate, however, suggests that component A may contribute the InGP combining site and component B the pyridoxylidine azomethine combining site to the surface on which reactions (1), (2), and (3) are catalyzed. Observations consistent with this hypothesis are the following: (1) the *td* series of mutants of *E. coli* represents a group in which alteration of component B[3] leaves the organism incapable of performing the reactions involving serine; (2) a component B-antibody complex can still participate in the conversion of InGP to indole while being essentially inactive in the pyridoxal phosphate–serine requiring conversion of indole to tryptophan;[3] and (3) mutational

alterations of component A seem to affect adversely the ability to act on InGP while permitting a limited conversion of indole to tryptophan.

Finally, it is of interest that similar studies performed in an attempt to detect an AB association in *N. crassa* have as yet been unsuccessful (Rachmeler, unpublished). Futhermore, neither component A nor component B from *E. coli* stimulates the activity of *N. crassa* TSase. Whether *N. crassa* TSase consists of two subunits, comparable to *E. coli* components A and B, which are linked by a peptide bond, or are associated more firmly, remains to be determined.

SUMMARY

E. coli tryptophan synthetase, the enzyme catalyzing the terminal step in the biosynthesis of tryptophan, was found to consist of two separable protein components. These two components combine with each other, and this combination seems to be responsible for the enzymatic activities detected.

The authors are greatly indebted to Mrs. Mary Hurd for her technical assistance and to Drs. M. Ahmad and D. G. Catcheside for *N. crassa* strain A78.

* This investigation was supported by grants from the National Science Foundation and the U.S. Public Health Service (RG-5652).

† Postdoctoral fellow, National Foundation for Infantile Paralysis.

[1] C. Yanofsky in W. D. McElroy and B. Glass, *Amino Acid Metabolism* (Baltimore: Johns Hopkins Press, 1955); see also n. 17.

[2] C. Yanofsky and M. Rachmeler, *Biochim. et Biophys. Acta*, **28**, 640, 1958.

[3] C. Yanofsky and J. Stadler, these PROCEEDINGS, **44**, 245, 1958; C. Yanofsky, *Biochim. et Biophys. Acta* (in press).

[4] C. Yanofsky, *J. Biol. Chem.*, **224**, 783, 1957.

[5] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265, 1951.

[6] C. Yanofsky, *J. Bact.*, **68**, 577, 1954.

[7] E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.*, **78**, 751, 1956.

[8] H. A. Sober, F. J. Gutter, M. M. Wyckoff, and E. A. Peterson, *J. Am. Chem. Soc.*, **78**, 756, 1956.

[9] P. Lerner and C. Yanofsky, *J. Bact.*, **74**, 494, 1957.

[10] Ultracentrifugal experiments were kindly performed by Dr. Fu-Chuan Chao.

[11] W. Umbreit, W. A. Wood, and I. C. Gunsalus, *J. Biol. Chem.*, **165**, 731, 1956.

[12] C. Yanofsky, in *Methods in Enzymology*, Vol. 2 (New York: Academic Press, 1955).

[13] Recently Richards (these PROCEEDINGS, **44**, 162, 1958) has presented evidence indicating that the enzyme subtilisin hydrolyzes a single peptide bond in ribonuclease, liberating a polypeptide 20 amino acid residues in length. Since both the liberated polypeptide and the residual protein are required for full enzymatic activity, this artificial two-component system resembles the AB interaction reported here.

[14] A. E. Braunstein and M. M. Shemyakin, *Doklady Akad. Nauk. SSSR*, **85**, 1115, 1952.

[15] A. E. Braunstein and M. M. Shemyakin, *Biokhimiya*, **18**, 393, 1953.

[16] D. Metzler, M. Ikawa, and E. E. Snell, *J. Am. Chem. Soc.*, **76**, 648, 1954.

[17] J. Harley-Mason, *Experientia*, **10**, 134, 1954.

[18] A. Braunstein, *Proc. Intern. Symposium Enzyme Chemistry* (Tokyo, 1957).

[19] E. L. Tatum and D. Shemin, *J. Biol. Chem.*, **209**, 671, 1954.

*DORMANCY OF BACTERIAL ENDOSPORES: REGULATION OF ELECTRON TRANSPORT BY DIPICOLINIC ACID**

By Harlyn Halvorson, Roy Doi and Brooks Church

DEPARTMENT OF BACTERIOLOGY UNIVERSITY OF WISCONSIN, MADISON, WISCONSIN AND WARNER-LAMBERT PHARMACEUTICAL CO., MORRIS PLAINS, NEW JERSEY

Communicated by Kenneth Raper, October 27, 1958

One of the most common features in the metamorphism of diverse biological systems is the conversion of these systems into a quiescent, dormant state. Although the properties of these dormant states have in general been well described (inert or weak metabolic activity and resistance to external chemical and physical factors), there is relatively little information on the internal regulatory mechanisms involved in either maintaining the dormant state or elevating its conversion to a metabolically active form.

In microbes these stages are well illustrated by the processes of sporulation and germination of bacterial endospores. The mature endospore is a highly refractile body which is resistant to heat and chemical agents and, although it contains several enzymes, is inert metabolically. Activation evokes a latent carbohydrate metabolizing system, a number of individual enzymes, and prepares the system for a triggered germination with loss of heat resistance upon the addition of simple germinating agents.[1] These observations, together with the dependence of over-all germination on metabolic activity,[2] suggest an enzymic regulatory mechanism for dormancy.

A priori one would expect this regulatory mechanism to reflect some fundamental chemical or biochemical difference between the dormant and vegetative states. Two such dramatic differences are the unique presence in spores of DPA[1, 2] and elevated levels of divalent metals.[4, 5] Our attention was directed toward these components as playing a role in dormancy, since (i) DPA synthesis and divalent metal uptake are essential for, and immediately precede, the production of resistant spores;[5, 6] (ii) activation of dormant spores is associated with the partial release into the medium of DPA salts;[7] (iii) germination parallels the complete release of DPA.[7, 8]

The recent demonstration that DPA stimulates the rate of oxygen uptake from glucose oxidation in spore extracts[9] and an analysis of the pathway of glucose metabolism[10] provided a basis for examining the regulatory role of DPA on spore metabolism. The present experiments were designed to classify further the basis of the DPA stimulatory effect.

MATERIALS AND METHODS

Spores of *Bacillus cereus* var. *terminalis* were used throughout this work. The methods employed for the preparation of washed spores, heat activation, and the preparation of spore extracts (1 gm spores/10 ml buffer) have been previously described.[2] To maintain reducing conditions during breakage of the spores, a steady stream of N_2 was included in the chamber of the Raytheon 10-kc. sonic oscillator. Unless otherwise indicated, 0.1 *M* glycylglycine, pH 7.3, was employed as buffer.

Oxygen uptake was measured by standard Warburg methods at 30°. Protein was determined by the method of Lowry *et al;*[11] using serum albumin as a standard. ATP and ADP hydrolysis was measured by following the rate of release of inorganic phosphate by the method of Fiske and SubbaRow.[12]

Diaphorase and cytochrome c reductase activities of the preparation were both measured spectrophotometrically at 30° with a DK2 recording spectrophotometer.[13] Diaphorase activity was followed by the rate of oxidation of DPNH by ferricyanide at 410 mμ and cytochrome c reductase by the rate of reduction of cytochrome c by DPNH at 550 mμ.

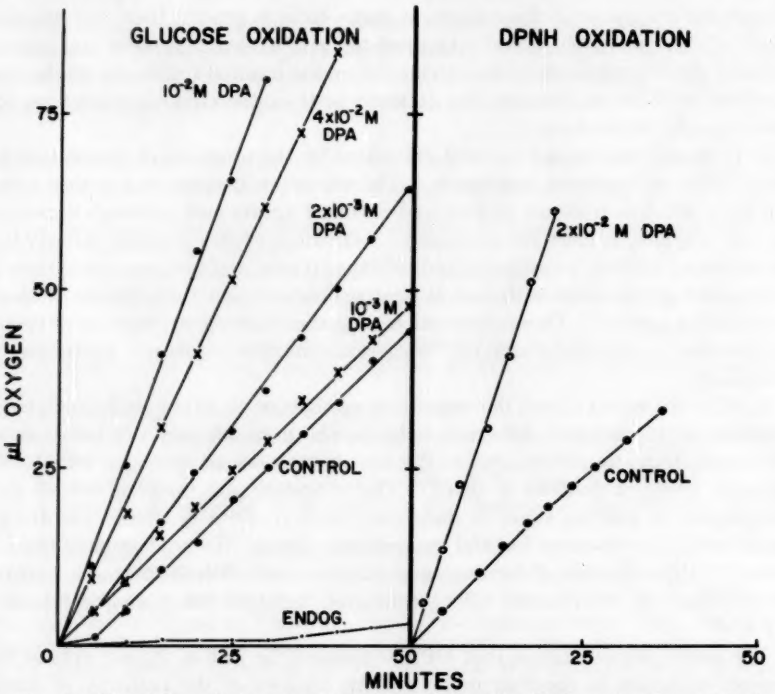


FIG. 1.—Stimulation of glucose oxidation by DPA. The Warburg vessels contained: glycylglycine buffer, pH 7.3, 75 μmoles; enzyme fraction with 4 mg. protein; DPA as indicated and (*a*) glucose, 20 μmoles; DPN, 0.7 μmoles; (*b*) DPNH, 10 μmoles. Final volume 1.8 ml. Center well contained 0.2 ml. of 20 per cent KOH. Incubation temperature, 30°.

In the assay for diaphorase activity the experimental cuvette contained 20 μmoles glycylglycine (pH 7.3), 0.5 μmole ferricyanide, 0.46 μmole of DPNH, enzyme and water to 1.0 ml. The control cuvette was identical except for the absence of enzyme. In the assay of DPNH cytochrome c reductase activity the final concentration of reactants in the 1-ml. cuvettes were as follows: 20 μmoles glycylglycine (pH 7.3), 0.46 μmole DPNH, and 0.05 μmoles of oxidized cytochrome c.

DPA was obtained from the Aldrich Chemical Company. The product was repeatedly (6 times) extracted from acid extracts with ether, and the concentrated preparation was crystallized from water. The resulting product gave the identical

spectra reported for DPA,[8] melted at 239° and stimulated oxygen uptake and DPNH cytochrome c reductase in a manner identical with that of the commercial product. ATP, ADP, AMP, DPN, DPNH, D-amino acid oxidase, and cytochrome c were products of the Sigma Chemical Company. Glycylglycine, EDTA, and 8-hydroxy quinoline were commercial samples.

RESULTS

Effect on DPA on Oxygen Uptake.—Harrell has observed that the addition of DPA to spore extracts leads to a twofold stimulation in the rate of oxygen uptake from the oxidation of glucose.[9] The results of a similar experiment, as seen in Figure 1, a,

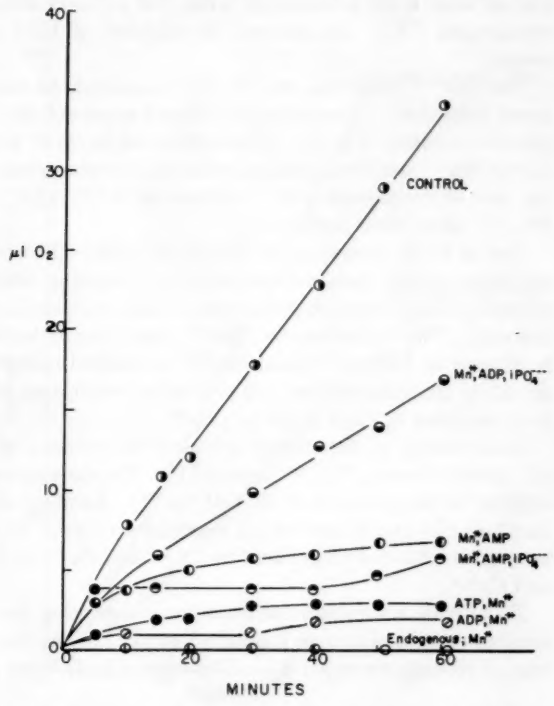


FIG. 2.—Reversal of Mn^{++} inhibition by ADP and inorganic phosphate. Spore extracts were dialyzed 12 hours against *M*/15 phosphate–*M*/150 EDTA buffer, pH 7.3, and 4 hours against *M*/20 glycylglycine buffer, pH 7.3. Warburg cup contents are identical with experiment in Fig. 1, *a*, except for $FeSO_4$, 1 μmole; $MnCl_2$, 100 μmoles; as indicated AMP, ADP, ATP, or $PO_4^{=}$, 5 μmoles.

show a threefold increase in the presence of 0.01 *M* DPA. Higher levels of DPA are slightly inhibitory. DPA is not metabolized by these extracts, nor is it stimulatory for the endogenous respiration.

Glucose oxidation by these extracts is mediated by a DPN-linked glucose dehydrogenase.[10] Since glucose dehydrogenase activity, as measured by the rate of DPNH formation, was found to be unaffected by the addition of DPA, the observed stimulation of O_2 uptake must occur in the electron-transport system. In support of this conclusion, the rate of oxygen uptake from DPNH is stimulated to the same extent by DPA as is glucose oxidation (Fig. 1, *b*).

Effect of Metallic Ions.—DPA forms chelate complexes with a number of divalent metals, including calcium, manganese, zinc, and copper. These findings, as well as

the stimulatory effect of another chelating agent, EDTA,[9] suggest that the observed stimulation may result from the chelation of some endogenous inhibitory metal. To test this possibility further, the sensitivity of the system to divalent ions was examined.

At 5×10^{-3} *M*, Fe^{++}, Fe^{+++}, Mg^{++}, and Ca^{++} enhanced glucose oxidation, whereas Mn^{++}, Ag^{+}, Co^{++}, Zn^{++}, Cu^{++}, Hg^{++}, and Pb^{++} inhibited oxygen uptake. The highest stimulations were observed by the addition of Fe^{++} (80 per cent) and Mg^{++} (42 per cent). Mn^{++} gave variable inhibitions of 60–100 per cent. Calcium (15–20 per cent stimulation at 5×10^{-3} *M*) is markedly inhibitory at concentrations above 2×10^{-2} *M*. Both the stimulatory and the inhibitory effects of metals were more pronounced when the extracts were first dialyzed to remove endogenous DPA. In general, the addition of DPA reversed the inhibition by metals.

The Mn^{++} inhibition was further examined, to characterize the locus of the metal inhibition. Employing a dialyzed spore extract, Mn^{++} completely inhibits glucose oxidation (Fig. 2). When substrate levels of ADP and PO_4^{---} were added to the Mn^{++} inhibited reaction mixture, oxygen uptake was again observed at 50 per cent of the normal rate. Additions of ATP, ADP, AMP, AMP + PO_4^{---} or PO_4^{---} alone were ineffective.

Test of DPA Control on the Supply of ADP.—The results in Figure 2 led us to speculate earlier[9] that in these extracts a coupling between the electron-transport system and oxidative phosphorylation may exist similar to that reported for other systems. The inhibition by Mn^{++} could readily be understood on the basis of a regulation of ATPase, whose activity is required to regenerate ADP from ATP for oxidation phosphorylation. A control of respiration of this type by calcium has been reported in heart preparations.[14]

Examination of the extract revealed the presence of an ATPase, pH optimum 8.1, which liberates PO_4^{---} from ATP. The dialyzed extract displays an optimum activity in the presence of 10^{-3} *M* Ca^{++}. Addition of 10^{-4} *M* Mn^{++} or 10^{-3} *M* Zn^{++} give 75 and 65 per cent of maximal activity. At 10^{-2} *M* concentrations, the enzyme is inhibited 45 per cent by Ca^{++} and Zn^{++} and over 90 per cent by Mn^{++} and Cu^{++}.

To provide a comparative basis for interpreting the respiration data, the subsequent experiments were conducted at pH 7.3 At this pH the enzyme has 53 per cent of its activity at pH 8.1. The extract hydrolyzes ADP and ATP at the same

TABLE 1

THE SUBSTRATE SPECIFICITY AND EFFECT OF DPA ON ATPASE*

Substrate	DPA	Specific Activity†
ATP	.....	0.35
ADP	.....	0.37
AMP	.....	0.023
ATP	3.2×10^{-2} *M*	0.42
ATP	8×10^{-3} *M*	0.46
ATP	1.6×10^{-3} *M*	0.40

* The systems for ATPase assay contained: glycylglycine buffer, pH 7.5, 100 μmoles; ATP, ADP, and AMP, 5 μmoles; DPA; and 2.0 ml. enzyme (16 mg.) in a final volume of 4.0 ml. Incubation for 60 min. at 30°. 2.0-ml. aliquots were taken at 0 and 60 min., deproteinized with 0.2 ml. of 50 per cent TCA, and centrifuged. 1.0 ml. of the supernatant was tested for PO_4^{---} by the Fiske-SubbaRow method. The results are corrected for endogenous PO_4^{---}.

† μM PO_4^{---}/mg protein/hr.

rate, while the rate of hydrolysis of AMP is at least 15 times slower than that of ATP (Table 1).

Based on the hypothesis that DPA regulates respiration by controlling the ADP level via its effect on ATPase, one would predict in crude extracts that (i) ATP hydrolysis would be stimulated two- to threefold by ADP, and ((ii) respiration would be dependent on ADP and PO_4^{---} levels. To test these possibilities, spores were heat-shocked, washed three times to remove endogenous phosphate, and cell-free extracts prepared. ATPase had a maximum stimulation of 30 per cent at 8×10^{-3} *M* DPA (Table 1).

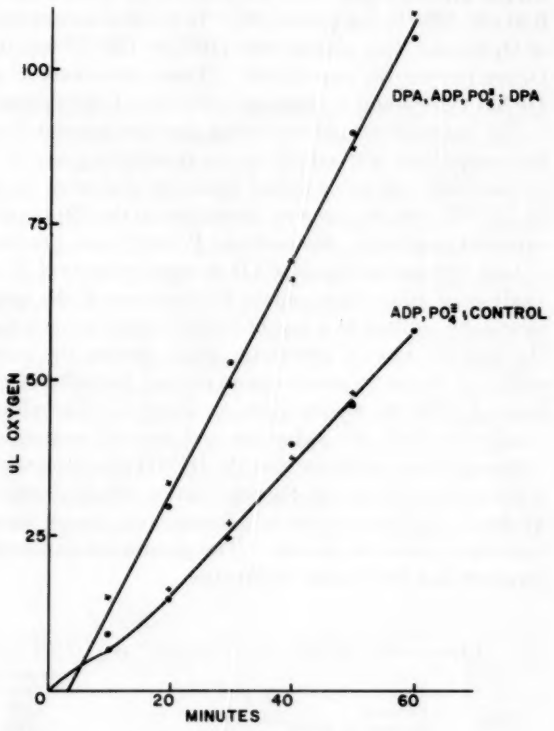


FIG. 3.—Independence of glucose oxidation from ADP. See Fig. 1, *a*, for details. Where indicated, ADP, $PO_4^{=}$, 5 μmoles; DPA, 20 μmoles.

Furthermore, the addition if ADP and PO_4^{---} to the extract did not significantly stimulate oxygen uptake from glucose (Fig. 3). The stimulation observed normally in the presence of DPA was unaffected by the presence of ADP and PO_4^{---}. Similar results were found either when DPNH was employed as substrate or when ATP substituted for ADP.

From these results it is clear that the major effect of DPA on oxygen uptake cannot be attributed to its effect on ATPase. The reason for the reversal of Mn^{++} poisoning by ADP and PO_4^{---} is not known at this time.

Effect of DPA on DPNH Cytochrome c Reductase.—Employing DPNH as substrate, a DPNH dehydrogenase was detected in extracts of heat-shocked spores.

The enzyme, which reacts with cytochrome c, ferricyanide, 2,6-dichlorophenol indophenol, and neotetrazolium as electron acceptors, may be similar to the diaphorase previously reported in spores and vegetative cells of *B. cereus* and *B. megaterium*.[1, 15] The enzyme is heat-labile and soluble. The activity is quantitatively recovered in the supernate fraction after centrifugation for 1 hour at 140,000 $\times$ *g*. in a Spinco preparation centrifuge.

The activity of the DPNH dehydrogenase, as well as over-all glucose oxidizing capacity, is variable in successive spore stocks. At 30° the diaphorase activity of the cell-free extracts, assayed by the ferricyanide method, varies from 3.5 to 5.3 μM DPNH/mg protein/hr, whereas the cytochrome c reductase activity is 0.23–0.50 μM DPNH/mg protein/hr. In these same extracts the variation in the rate of O_2 uptake from glucose and DPN or DPNH was 0.15–0.40 and 0.20–0.51 μM O_2/mg protein/hr, respectively. These rates were in general agreement with the DPNH cytochrome c reductase activities of the various extracts.

The enzymes usually mediating electron transfer from DPNH to cytochrome c have some form of riboflavin as the prosthetic group.[13] Spectroscopic examination of the spore extract, reduced either by dithionite or under anaerobic conditions by DPNH, showed a general absorption in the 450-mμ region where flavins would be expected to absorb. Spencer and Powell[15] have previously reported the presence of both free and combined FAD in spore extracts of *B. subtilis* and *B. megatherium*. Dialysis or ammonium sulfate fractionation of the spore extracts of *B. cereus* var. *terminalis* resulted in a loss of DPNH cytochrome c reductase activity, indicating the possible loss of prosthetic group during the procedures. Accordingly, the ability of flavins to reactivate an extract partially inactivated by dialysis was examined, with the results given in Table 2. The addition of FMN to the extract reactivates both the reductase and over-all respiration. FAD is less effective. Although direct evidence that the DPNH cytochrome c reductase of these spores is a flavoprotein is lacking, the reactivation obtained with added FMN is presumptive evidence that this enzyme is a flavoprotein, as are the other DPNH cytochrome c reductases examined to date. The purification and properties of the enzyme will be reported in a subsequent publication.

TABLE 2

REACTIVATION OF OXIDATIVE CAPACITY* AND DPNH CYTOCHROME c REDUCTASE†

	Rate of O_2 Uptake Per Cent Control	Reductase Per Cent Control
Extract	100	100
Extract + FAD	104	140
Extract + FMN	148	600
Extract + FAD + FMN	153	600

* See Fig. 1, *a*, for details. Where indicated FAD, 25 mμmoles; FMN 39 mμmoles.

† Reductase activity was determined as described under "Materials and Methods" except for the addition of FAD, 25 mμmoles and FMN, 39 mμmoles. The co-factors were added to both the reference and the test cuvettes.

Table 3 shows the effect of DPA addition on the diaphorase and DPNH cytochrome c reductase activities. The dehydrogenase, extracted from both heat-shocked spores and vegetative cells, is stimulated by DPA to approximately the same degree as over-all oxygen uptake with either glucose or DPNH as substrates (Fig. 1, *a*, *b*). When the soluble enzyme is further purified by ammonium sulfate precipitation and fractionation by DEAE cellulose chromatography, the resulting

preparation retains both its DPA-enhanced diaphorase and DPNH cytochrome c reductase activities. The locus of a DPA effect at the DPNH cytochrome c reductase level is further supported by the fact that in extracts of heat-shocked spores, the rate of cytochrome c reduction by glucose and DPN is stimulated threefold by 0.02 *M* DPA.

TABLE 3

STIMULATION OF DPNH OXIDATION BY DPA

SOURCE OF EXTRACT	REDUCTASE*		DIAPHORASE*	
	Control	DPA†	Control	DPA
Heat-shocked spores	0.23	0.90	4.9	13.2
Vegetative cells	0.98	3.32	33.8	96

* μM DPNH/mg protein/hr.
† 0.02 *M*.

Table 4 summarizes experiments designed to determine whether the stimulation of DPNH cytochrome c reductase could be ascribed to the removal of an inhibitory agent by chelation with DPA. A survey of chelating agents on the activity of the soluble enzyme indicates that the stimulatory phenomenon is specific for DPA. One of these agents—8 hydroxyquinoline—has no effect on the system, whereas EDTA inhibits DPNH cytochrome c reductase activity. Dialysis of the extract from heat-shocked spores against glycylglycine buffer leads to a 40–50 per cent reduction of enzymatic activity, probably because of a loss of flavins. A similar loss in activity occurs when the preparation is first dialyzed for 12 hours against DPA, EDTA, or 8 hydroxyquinoline and then dialyzed against buffer to remove the chelating agents. In none of the dialyzed preparations has the DPA effect been abolished. Also, when care was taken to decrease the divalent metal content of extracts of vegetative cells which had either been grown in a medium from which Zn^{++}, Mn^{++}, or Ca^{++} were withheld, disrupted in non-metal containers (freeze and thawing, Nossal disintegrator, or Hughes's press), or assayed with DPA-dialyzed cytochrome c, a threefold increase in reductase was observed in the presence of 0.02 *M* DPA.

TABLE 4

EFFECT OF CHELATING AGENTS ON DPNH CYTOCHROME C REDUCTASE*

DIALYSIS‡	INCUBATION CONDITIONS†				DPA/ Control
	Control	DPA	Versene	8-OHQ§	
None	2.28	6.67	1.40	2.28	2.9
Buffer	1.61	4.92	0.95	..	3.0
DPA; buffer	1.08	3.79	..	..	3.5
Versene; buffer	1.12	2.73	..	..	2.2
8-OHQ; buffer	1.30	4.21	..	1.30	3.2

* μM DPNH/ml./hr.
† Reductase activity was determined as described in methods except for the addition of 10^{-2} *M* chelating agents where indicated.
‡ Dialyzed at 2° for 12 hours against 10^{-2} *M* chelating agent, followed by 12 hours against 3 changes of 10^{-2} *M* glycylglycine buffer, pH 7.3.
§ 8 hydroxy quinoline.

The effect of DPA on a known flavoprotein, the D-amino acid oxidase of hog kidney, was also examined. Employing DL-alanine as substrate with added catalase and FAD,[16] the rate of oxidation of D-alanine was unaffected by the presence of 0.02 *M* DPA.

DISCUSSION

Although DPA has been recognized as a major constituent of bacterial endospores since 1953,[8] little is presently known about either its state or its function in dormant spores. The DPA content of spores is not constant; for example, variations of 3–6 per cent for *B. cereus* var. *mycoides*,[17] 6–12 per cent for *B. cereus* var. *terminalis*,[1, 7] and 5–15 per cent for *B. megatherium*[8, 17] have been reported. The use of different assay methods for DPA may explain some of these variations; however, modifications of the sporulation medium greatly influences the DPA content of spores.[18] Knowledge of the state of DPA in dormant spores is equally incomplete. The isolation of the Ca DPA from germination exudates[8] and the demonstration that DPA strongly chelates Ca^{++}, Cu^{++}, Fe^{++}, and Mn^{++} and weakly chelates Mg^{++}[5] have led to various suggestions that DPA exists in spores in the chelate form. That DPA is distributed within the resting spore exclusively as the calcium salt is made unlikely on several grounds: (i) air-dried spores are devoid of the characteristic crystalline-powder X-ray diagram of calcium DPA;[5] (ii) Ca^{++}/DPA ratios of 0.021[19] to over 1[5] have been reported; (iii) hydrolysis by hot mineral acids is required to obtain total recoveries of DPA.[7, 17]

Although the mineral contents of spores are variable and to a certain extent the ions are interchangeable,[4] the need for acid hydrolysis to liberate free DPA implies that in dormant spores DPA exists in part in a bound inorganic or organic form. One such form is suggested by the isolation of the monethyl ester of DPA from the hydrolyzates of intact spores.[17] Although the nature and extent of the bound DPA forms are unknown, it seems clear from the above and from the eventual total liberation of DPA during germination and outgrowth[1, 7, 20] that the full chelation potential of DPA has not been realized in dormant spores.

It is not surprising that the introduction of a strong chelating agent in vivo or in vitro influences the metal balance and over-all metabolism of a system known to contain both metal-activated and metal-sensitive enzymes.[1] The stimulation of respiration from glucose in spore extracts upon the addition of either DPA or EDTA, as reported by Harrell[9] or in this paper, is undoubtedly due to their chelating properties. The system can be poisoned by many of the divalent metals known to be present in spores in high concentrations;[4, 5] the inhibition is overcome by the addition of DPA or EDTA. On the other hand, the metal requirement of this same system is further suggested by the stimulatory effect of 5×10^{-3} *M* Fe^{++}, Mg^{++}, and Ca^{++}. One would expect that, in the presence of excess free DPA, Ca^{++}, Cu^{++}, Ni^{++}, Fe^{++}, and Mn^{++} would be displaced from enzymes whose chelation stability constants for these metals was less than their affinities for DPA. Although undoubtedly various degrees of metal competition may exist between DPA and spore proteins, the slight stimulation reported here with a Ca^{++}-activated ATPase is insufficient to explain the over-all DPA effect in electron transport. The failure of DPA to inhibit ATPase suggests that the enzyme may largely retain its activating metal in the presence of DPA. Further studies on DPA effects on metal binding proteins, e.g., Mn^{++}-activated spore enzymes,[1] would seem warranted.

A second and perhaps more prominent function of DPA in electron transport, aside from the removal of inhibitory metals, is suggested by its effect on DPNH cytochrome c reductase. The enzyme, which had previously been dialyzed against several chelating agents, is specifically stimulated threefold by DPA and inhibited

by EDTA. This phenomenon is not unique to the dormant state, since similar observations were obtained with DPNH cytochrome c reductase of vegetative cells. Numerous models could be proposed to explain the DPA effect on this enzyme; however, an explanation of this phenomenon must await an analysis of the DPA-enzyme interaction.

The inhibition of DPNH cytochrome c reductase by EDTA clearly distinguishes this phenomenon from the stimulatory effect of EDTA on over-all oxygen uptake. It would thus appear that at least two sites are involved in the over-all stimulation of electron transport: (*a*) a site which is stimulated, probably via chelation, by EDTA and DPA and (*b*) a DPA specific stimulation. Until the nature of the native cytochromes and terminal respiratory systems of these spores are further characterized, it will be difficult to quantitate the contribution of these DPA-sensitive sites on regulating over-all electron transport.

The demonstration that DPA regulates electron transport in spore extracts suggests that it may play an important role in dormancy. An integrated biochemical description of dormancy demands a basis for converting the vegetative cell into the heat-resistant, quiescent state, as well as maintaining the dormant state. Of the various factors involved, heat resistance is correlated with the contents of Ca^{++},[4] DPA,[18] and possibly water,[20, 21] while the breaking of dormancy is stimulated by activation[1, 2] and by metabolizable germinating agents,[1, 2, 10] is dependent on metabolic activity,[2] and is sensitive to divalent metals.[1, 22] The early stages of germination are associated with the release of DPA,[1, 7] the appearance of respiratory activity, and the liberation of a number of dormant enzymes.[1]

The foregoing findings, the high content of divalent metals in spores,[4, 5] and the necessity of activating the electron-transport system of dormant spores for germination have suggested to us the following model for the regulatory role of DPA in electron transport. Activation leads to an increase in chelation potential by releasing bound forms of DPA. The liberated free DPA promotes the flow of electrons from substrate to molecular O_2 by (1) the removal of an inhibitory metal directly or indirectly affecting the system and by (2) a direct stimulation of DPNH cytochrome c reductase. We are currently attempting to test some of the predictions afforded by such a model.

SUMMARY

The rate of oxygen uptake from glucose or DPNH by cell-free extracts of *B. cereus* var. *terminalis* is stimulated by the addition of free dipicolinic acid and versene. Glucose oxidation was inhibited by Mn^{++}, Ag^{+}, Co^{++}, Zn^{++}, Cu^{++}, Hg^{++}, and Pb^{++}, the inhibition being reversed by DPA. Mn^{++} inhibition was reversed by ADP and inorganic phosphate.

A Ca^{++}-activated ATPase was detected in spore extracts whose activity was stimulated 30 per cent by 8×10^{-3} DPA. Attempts to demonstrate a dependence of respiration on ATPase activity or ADP levels was negative.

A soluble DPA-stimulated DPNH cytochrome c reductase was extracted from spores and vegetative cells. The enzyme is inhibited by versene and unaffected by 8 hydroxyquinoline. The specific stimulatory effect of DPA could not be attributed to the removal of an inhibitory ion.

The possible role of divalent ions and DPA in regulating the respiratory dormancy of spores is discussed.

* This investigation was supported in part by a research grant of the Wisconsin Foundation and by an institutional grant of the American Cancer Society. The authors wish to acknowledge the technical assistance of Mr. Ray Epstein and the helpful advice of Dr. Salih Wakil. The early phases of this program were conducted in co-operation with Dr. W. K. Harrell.

[1] For a review of the literature see W. G. Murrell, *The Bacterial Endospore* (Sydney, Australia: University of Sidney, 1955), pp. 1–79; C. F. Schmidt, *Ann. Rev. Microbiol.*, **9**, 387, 1955; R. L. Stedman, *Am. J. Pharm.*, **128**, 84, 1956; H. Halvorson and B. D. Church, *Bacteriol. Revs.*, **21**, 112, 1957; H. O. Halvorson, *Spores* (New York: Amer. Inst. of Biol. Sci., 1957), p. 120.

[2] H. Halvorson and B. D. Church, *J. Appl. Bacteriol.*, **20**, 359, 1957.

[3] The following abbreviations are used in this paper: AMP, adenosine-5′-phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPA, dipicolinic acid; DPNH, dihydrodiphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetic acid; FMN, riboflavin-5′-phosphate; FAD, flavin adenine dinucleotide; DEAE cellulose, diethyl-aminoethyl cellulose.

[4] H. R. Curran, B. C. Brunstetter, and A. T. Myers, *J. Bacteriol.*, **45**, 485, 1943; R. A. Slepecky and J. W. Foster, *Bacteriol. Proc.*, p. 43, 1958.

[5] J. F. Powell and R. E. Strange, *Biochem. J.*, **63**, 661, 1956.

[6] H. O. Halvorson, *J. Appl. Bacteriol.*, **20**, 305, 1957.

[7] W. K. Harrell and E. Mantini, *Can. J. Microbiol.*, **3**, 735, 1957.

[8] J. F. Powell, *Biochem., J.*, **54**, 210, 1953.

[9] W. K. Harrell, R. Doi, and H. Halvorson, *J. Appl. Bacteriol.*, **20**, xiii, 1957; W. K. Harrell, *Can. J. Microbiol.*, **4**, 393, 1958.

[10] R. Doi, H. Halvorson and B. D. Church, *J. Bacteriol.* (in press).

[11] O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265, 1951.

[12] C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375, 1925.

[13] A. Edelhoch, O. Hayaishi, and L. J. Teply, *J. Biol. Chem.*, **197**, 97, 1952; B. De Bernard, *Biophys. Acta*, **23**, 510, 1957.

[14] E. C. Slater, *Third Intern. Cong. Biochem.* (Brussels, 1955), p. 264.

[15] R. E. Spencer and J. Powell, *Biochem. J.*, **51**, 239, 1952; N. Kunita and T. Fukumaru, *J. Osaka Univ.*, **6**, 955, 1956.

[16] H. R. Mahler, *Methods in Enzymology* (New York: Academic Press, Inc.), **2**, 688.

[17] J. J. Perry and J. W. Foster, *J. Bacteriol.*, **72**, 295, 1956.

[18] H. H. Martin and J. W. Foster, *J. Bacteriol.*, **76**, 167, 1958; B. D. Church and H. Halvorson, in preparation.

[19] J. J. Perry and J. W. Foster, *Texas Repts. Biol. and Med.*, **13**, 920, 1955.

[20] J. F. Powell and R. E. Strange, *Biochem. J.*, **54**, 205, 1953.

[21] W. G. Murrell and W. J. Scott, *Abstracts 7th Intern. Cong. Microbiol.* (Stockholm, 1958), p. 26.

[22] G. G. K. Murty and H. O. Halvorson, *J. Bacteriol.*, **73**, 230, 1957.

---

*OBSERVATIONS ON THE FUNCTION OF PYRIDOXAL-5-PHOSPHATE IN PHOSPHORYLASE**

BY BARBARA ILLINGWORTH, HENDRIK S. JANSZ†, DAVID H. BROWN, AND CARL F. CORI

DEPARTMENT OF BIOLOGICAL CHEMISTRY, WASHINGTON UNIVERSITY SCHOOL OF MEDICINE, ST. LOUIS, MISSOURI

Communicated October 2, 1958

The discovery that 4 moles of pyridoxal-5-phosphate are present per mole of muscle phosphorylase *a* and that this substance is the prosthetic group of the enzyme[1, 2] raises questions as to (1) its possible direct participation in the enzymatic reaction, (2) its mode of attachment to the protein, and (3) its importance

in maintaining the catalytically active structure of the protein. The first question has been studied with isotopically labeled substrates and phosphorylase *a*. The second and third problems have been approached by improving the methods of resolution of phosphorylases *a* and *b* and by studying spectrophotometrically and ultracentrifugally the interaction of pyridoxal-5-phosphate itself and of some structural analogues of it with the apoenzymes. This work has led incidentally to the crystallization of apophosphorylase *a*.

Experiments on the Mechanism of the Phosphorylase Reaction.—In 1948 Cohn and Cori showed that no exchange occurs between inorganic phosphate and glucose-1-phosphate when these are incubated with muscle phosphorylase *a* in the absence of glycogen.[3] It was also shown that no exchange occurs between free glucose and either glucose-1-phosphate or glycogen when the phosphorylase *a* reaction reaches equilibrium in the presence of glycogen. In the present work the lack of exchange between glucose-1-phosphate and inorganic phosphate in the absence of glycogen was confirmed in an experiment in which 114 mg. of four times crystallized phosphorylase *a* (0.23 μmole) were incubated at 30°, pH 7.5, for 20 minutes with P^{32}-inorganic orthophosphate and C^{14}-glucose-1-phosphate. Following deproteinization with trichloroacetic acid and extraction of the solution with ethyl ether, an aliquot of the solution, containing 5.31 μmoles C^{14}-glucose-1-phosphate (*sp. act.*, 12,080 cpm/μmole) and 8.37 μmoles P^{32}-inorganic phosphate (*sp. act.*, 8,660 cpm/μmole), was adsorbed on a 23 × 1 cm., 8 per cent cross-linked, Dowex-1 (formate) column. Development was by gradient elution with 1 *M* ammonium formate, using 500 ml. of water in the mixing chamber. After 65 fractions of 2.8 ml. each had been collected, the C^{14}-glucose-1-phosphate emerged as a symmetrical peak (fractions 66–71) containing 4.53 μmoles (85 per cent recovery) with an average specific activity of 11,300 cpm as C^{14}. The absence of P^{32} was established with an end-window counter, using an aluminum-foil shield which was found to pass 80 per cent of the counts from a P^{32} standard and no counts from a C^{14}-glucose-1-phosphate standard. The inorganic phosphate was present in a peak (fractions 72–76) containing 8.48 μmoles (101 per cent recovery) with an average specific activity of 7,380 cpm as P^{32}.

The dependence of P^{32} exchange on the presence of glycogen leaves open the possibility that pyridoxal-5-phosphate participates directly in the enzymatic reaction, as follows, where Enz-Pyr_5-P represents one or more of the four molecules of pyridoxal-5-phosphate which are bound to phosphorylase *a*[4]:

$$G_1\text{-}P^{32} + \text{Enz-}Pyr_5\text{-P} = \text{Enz-}Pyr_5\text{-}P^{32}\text{-}_1G + P_{inorg} \quad (1)$$

$$\text{Enz-}Pyr_5\text{-}P^{32}\text{-}_1G + (\text{glucose})_n = (\text{glucose})_{n+1} + \text{Enz-}Pyr_5\text{-}P^{32} \quad (2)$$

At equilibrium, in the presence of added glycogen, at least some of the pyridoxal-5-phosphate bound to the enzyme would be labeled with P^{32} if, initially, either P^{32}-glucose-1-phosphate or inorganic P^{32} were added to the incubation medium. Accordingly, the following experiment was done.

70.2 mg. of four times crystallized phosphorylase *a* were dissolved in 5.0 ml. of 0.01 *M* imidazole buffer, pH 7.6. This amount of enzyme contained 0.57 μmole of bound pyridoxal-5-phosphate. The enzyme solution was mixed with 1.0 ml. of a solution at pH 7.4 containing 11.2 μmoles of uniformly labeled C^{14}-glucose-1-phosphate (66,815 cpm),[5] 4.4 μmoles of P^{32}-inorganic orthophosphate (316,000

cpm), and 1.6 mg. of glycogen. The reaction mixture was incubated for 30 minutes at 30°, and 1.0 ml. of 40 per cent trichloroacetic acid was then added. As soon as the transient yellow color of the protein was discharged, the mixture was cooled in ice, centrifuged in the cold, and the supernatant fluid kept at 0°. The precipitated protein was washed in the cold with two portions of 5 per cent trichloroacetic acid, and the washings were combined with the original supernatant fluid. This solution was thoroughly extracted with ethyl ether, and the aqueous phase was adjusted to pH 6.6 and diluted to exactly 10.0 ml. Chemical analysis of this solution showed the presence of 12.7 μmoles of inorganic phosphate and of 4.4 μmoles of glucose-1-phosphate. The observed molar ratio of 2.9 for these substances shows that the phosphorylase reaction had come to equilibrium during the incubation period.[6] Spectral analysis of the trichloroacetic acid extract showed the presence in it of 0.43 μmole of pyridoxal-5-phosphate, which corresponded to the recovery of 75 per cent of this substance from the enzyme.

To 9.0 ml. of the trichloroacetic acid extract, containing 11.4 μmoles of inorganic phosphate, 3.96 μmoles of glucose-1-phosphate, and 0.39 μmole of pyridoxal-5-phosphate were added 2.0 μmoles of pure, unlabeled pyridoxal-5-phosphate in order to increase the content of this component (sixfold dilution of the pyridoxal-5-phosphate from phosphorylase). The solution was adjusted to pH 8.2 and adsorbed on a 10 × 1 cm., 8 per cent cross-linked, Dowex-1 (formate) column. Development was by gradient elution with 2 *N* HCOOH, using 500 ml. of water in the mixing chamber. After 77 fractions of 2.8 ml. each had been collected, the eluent was changed to 4 *N* HCOOH. The pyridoxal-5-phosphate peak was found by direct spectrophotometric assay at 293 mμ. This substance was in fractions 59–68, which contained 1.15 μmoles (48 per cent recovery). Aliquots (0.2 ml.) of the individual fractions were plated and first counted for P^{32} in an end-window counter. These planchets had radioactivity indistinguishable from background, which was 24 cpm. In addition, the fractions were counted in a windowless, gas-flow counter in which any C^{14} as well as any P^{32} would have been detected. All fractions had from 1 to 3 cpm above background, which was 22 cpm (counted for 640 total counts). However, the net count was independent of the pyridoxal-5-phosphate content of the fractions. It was apparent that the C^{14}-glucose-1-phosphate on the column had slowly been hydrolyzed by the acidic eluent and that traces of C^{14}-glucose were present in all fractions. The peak tubes containing pyridoxal-5-phosphate were pooled, and the pool concentrated *in vacuo* to one-tenth its initial volume. An aliquot of this solution was plated, which contained 0.014_3 μmole of pyridoxal-5-phosphate from phosphorylase. Had the phosphate group of each of the four molecules of pyridoxal-5-phosphate present in phosphorylase become labeled with P^{32} during the enzymatic incubation, the planchet would have had 247 cpm as P^{32}. This planchet had only 2.7 cpm above background. Thus no more than 1 per cent exchange of P^{32} from inorganic phosphate could have taken place into the four moles of pyridoxal-5-phosphate present in phosphorylase when the enzymatic reaction with glycogen was at equilibrium. That there were no unidentified C^{14} or P^{32} counts present in the pyridoxal-5-phosphate peak was shown by comparing in a windowless, gas-flow counter the planchet prepared from the concentrate of the peak with planchets prepared from concentrates of column fractions taken before and after the pyridoxal-5-phosphate peak. Each

of these planchets had 9 cpm above background per milliliter of column fraction. Thus this radioactivity appeared to be due only to free C^{14}-glucose, as mentioned above.

In view of the absence of P^{32} in the pyridoxal-5-phosphate from the enzyme, it was important to establish that in this experiment glucose-1-phosphate and inorganic phosphate had equal specific activities with respect to P.[32] 3.64 μmoles of glucose-1-phosphate were recovered (92 per cent of theory) from column fractions 107–123. The ester in these fractions had an average specific activity of 16,060 cpm of P^{32} per μmole. 10.4 μmoles of inorganic phosphate were recovered (91 per cent of theory) from column fractions 148–177. In various fractions its average specific activity was 17,310 cpm per μmole. An aliquot of the trichloroacetic acid extract of the enzyme counted at the same time had 16,500 cpm of P^{32} per μmole of inorganic phosphate plus glucose-1-phosphate. Thus the phosphorylase reaction mixture was substantially at equilibrium at the time the incubation was stopped with trichloroacetic acid.

From this experiment it may be concluded that the phosphate group of the pyridoxal-5-phosphate in phosphorylase is never in equilibrium with inorganic phosphate in the course of the enzymatic reaction catalyzed by the enzyme. Thus, no support was found for the mechanism described by equations (1) and (2).

Resolution of Phosphorylase a.—A readily soluble, resolved enzyme could be obtained in crystalline form by the following procedure: 2–5 ml. of a phosphorylase *a* suspension (sixth crystals in 0.001 *M* EDTA–0.02 *M* glycerophosphate, pH 6.7) were centrifuged in the cold, and the crystalline precipitate was dissolved in 0.05 *M* $NaHCO_3$ (5–10 mg protein/ml). The solution was made 0.05 *M* in cysteine by the addition of freshly neutralized 0.2 *M* cysteine and then was chilled. An equal volume of ice-cold, 0.2 *M* Na citrate, pH 3.5 was added, and the mixture was allowed to stand 3–5 minutes in ice prior to the addition of saturated $(NH_4)_2SO_4$,[7] pH 8.0–8.5, to 60 per cent saturation. Five minutes later, the mixture was centrifuged in the cold. The supernatant fluid was pH 4.8–5.0 (direct reading in the cold using a Beckman pH meter set at 10°). The precipitated protein was washed twice with cold, pH 8.9, 50 per cent saturated $(NH_4)_2SO_4$ in 0.2 *M* Tris buffer. When the protein was dissolved in freshly prepared 0.05 *M* Na thioglycolate, pH 7.4, and dialyzed in the cold overnight against the same solution, the apoenzyme crystallized.[8] Often, only a small granular precipitate was present initially in the dialysis bags, but when the solution was agitated or the sides of a vessel containing the solution were scratched, a heavy, white, crystalline precipitate appeared. The apoenzyme could be recrystallized by centrifuging the crystals, dissolving them in a fresh portion of thioglycolate buffer at room temperature, and chilling the solution. For good recovery of the protein it was necessary to allow the crystallization mixture to stand 24 hours in the cold. The specific activity of the apoenzyme (in the presence of a saturating amount of added pyridoxal-5-phosphate) was from 1,000 to 1,900 units[6] per milligram protein, depending upon the initial activity of the unresolved enzyme and was not changed in any one preparation during the course of several recrystallizations. Figure 1 shows a photomicrograph of the crystals obtained.

Ultracentrifugal Properties of Apophosphorylase a.—For ultracentrifugal study the resolved enzyme was dissolved in and dialyzed for 2 hours in the cold against

FIG. 1.—Photomicrograph under phase contrast of crystals of apophosphorylase *a*. ×430.

0.03 *M* cysteine –0.02 *M* glycerophosphate buffer, pH 7.3. Unlike phosphorylase *a*, which crystallized under these conditions, the resolved enzyme was soluble and stable as long as the pH did not become lower. In the ultracentrifuge two principal peaks were observed—one, corresponding to about 40 per cent of the total with $s_{20}' = 4.7$ to 5.8 (uncorrected for salt) and the other corresponding to about 60 per cent of the total with $s_{20}' = 12.0$ to 12.2 (uncorrected for salt). When phosphorylase *a* was subjected to the resolution procedure except for the omission of the acid citrate buffer, it maintained full enzymatic activity in the absence of added pyridoxal-5-phosphate and showed a single sharp peak in the ultracentrifuge, $s_{20}' = 12.1$. Thus the resolved enzyme appears to be a mixture of monomer units similar to those found by Madsen and Cori,[9] together with tetramer units which are identical in size with the complete phosphorylase *a*. The amount of any dimer or other units which may be present is difficult to estimate because the monomer and tetramer peaks observed do not become sufficiently discrete during the time of the run. However, the degree of spreading of the pattern suggests that some molecules of other sizes may, in fact, be present.

Table 1 shows that incubation with an equivalent amount of pyridoxal-5-phosphate converted the ultracentrifugal pattern of the resolved enzyme completely into that of a tetramer unit ($s_{20}' = 12.2$). Of particular interest was the finding that incubation of the apoenzyme with a large excess of pyridoxal or of 5-deoxypyridoxal[10] gave a protein indistinguishable in the ultracentrifuge from phosphorylase *a* or from apophosphorylase *a* plus pyridoxal-5-phosphate. However, the proteins resulting from incubation with the non-phosphorylated analogues had recovered no enzymatic activity. On the other hand, pyridoxamine-5-phosphate, even in

TABLE 1

INTERACTION OF APOPHOSPHORYLASE *a* WITH DERIVATIVES OF PYRIDOXAL

Experiment	Combining Sites of Apoenzyme ($M \times 10^6$)	Substance Added during Preincubation* ($M \times 10^6$)	Enzymatic Activity† (Units/Mg Protein)	Observed ss' of Peaks in Ultracentrifuge
A	4.8	None	234	5.8, 12.2
	4.8	Pyr_5-P, 19	1,154	12.2
B	5.2	None	117	4.7, 12.1
	0.48	Pyr_5-P, 0.29	548	....
	5.2	Pyr_5-P, 5.6	770	12.0
	0.48	Pyr_5-P, 1.2	1,145	....
C	4.8	None	60	4.9, 12.0
	4.8	Pyridoxal, 100	66	12.2
	0.48	Pyr_5-P, 1.5	1,000	....
D	0.64	None	66	....
	4.4	Pyridoxamine-5-P, 100	82	5,6. 12.0
	0.64	Pyr_5-P, 90	705	....
E‡	4.4	None	57	7.1, 13.4
	0.20	Pyr_5-P, 1.0	960	....
	4.4	5-Deoxypyridoxal, 100	96	13.3
F‡	4.0	None	80	7.3, 14.0
	0.68	Pyr_5-P, 0.67	970	....

* Preincubated in 0.01 *M* cysteine–0.02 *M* glycerophosphate, 20 minutes, pH 7.5, 30°.
† Assayed in presence of cysteine and AMP-5′, pH 6.7 (Cori and Illingworth, these PROCEEDINGS, **43**, 547, 1957).
‡ Crystalline apoenzyme used.

high concentrations, had no ability to alter the ultracentrifugal pattern of apophosphorylase *a*.

These experiments prove that the combination of pyridoxal-5-phosphate with the protein in phosphorylase *a* is through its 4-formyl group and that the nature of the side chain at position 5 of the pyridine ring has no apparent influence on the association of the monomer units into the tetramer form characteristic of the complete enzyme.

Information about the nature of the linkage of the 4-formyl group to the protein was obtained from experiments with the carbonyl reagent, isonicotinic acid hydrazide.[11] No inhibition of phosphorylase *a* activity occurred when the enzyme was incubated with a 0.02 *M* solution of the hydrazide. On the other hand, combination of apophosphorylase *a* with pyridoxal-5-phosphate could be prevented by preincubation with the carbonyl reagent. Thus, when isonicotinic acid hydrazide at 0.05 *M* was incubated with 4×10^{-5} *M* pyridoxal-5-phosphate at pH 7, and the mixture then diluted and added to the apoenzyme, the activity was only 197 units/mg protein. In a control experiment without preincubation with the carbonyl reagent, the activity was 1,145 units/mg protein. These findings show that the mode of union of pyridoxal-5-phosphate with the protein is not as a simple Schiff base which would be dissociated in the presence of a large excess of the carbonyl reagent. Kent *et al.*[12] reached a similar conclusion using phosphorylase *b* by studying the spectral properties of the enzyme.

Interaction of Pyridoxal and of 5-Deoxypyridoxal with Apophosphorylase a.— As discussed above, the data in Table 1 show that pyridoxal and 5-deoxypyridoxal are able to restore apophosphorylase *a* to the molecular size of phosphorylase *a*. Direct evidence that these substances actually combine with the protein at the same or at a closely neighboring site to that which pyridoxal-5-phosphate would occupy was obtained by preincubating the apoenzyme with the nonphosphorylated derivatives, and then incubating it with pyridoxal-5-phosphate. Table 2 shows

that 5-deoxypyridoxal was more effective than pyridoxal in preventing the combination of the apoenzyme with pyridoxal-5-phosphate and that the extent of inhibition by either substance depended upon its concentration. In other experiments not included in the table, preincubation with pyridoxamine-5-phosphate had no effect on subsequent activation by pyridoxal-5-phosphate. The affinity of the enzyme is considerably greater for pyridoxal-5-phosphate than for 5-deoxypyridoxal, as shown by the small degree of inhibition observed when the two substances were added simultaneously to the apoenzyme. The data in Table 2 also show that the analogues of pyridoxal-5-phosphate combine with the apoenzyme to give other than Schiff-base structures, since the combination is not reversed by subsequent addition of pyridoxal-5-phosphate.

Reaction of ω-Methylpyridoxal-5-Phosphate with Apophosphorylase a.—The 2-methyl analogue of pyridoxal-5-phosphate was synthesized by Ikawa and Snell[13] and found to be partially active in the alanine racemase and in various transami-

TABLE 2

INHIBITION OF REACTIVATION BY PYRIDOXAL-5-PHOSPHATE OF APOPHOSPHORYLASE *a*

Substance Preincubated* with 1.4×10^{-4} *M* Apoenzyme	Concentration ($M \times 10^6$)	Activity (Units/Mg Protein)
None		1,230, 1,225, 1,300
5-Deoxypyridoxal	100	85
	50	177
	10	318, 382
	5	535
	1	693
	0.5	955
5-Deoxypyridoxal + Pyr_5-P	10 + 1.6	955
Pyridoxal	120	260
	12	500
	1.2	1,060

* Apophosphorylase *a* preincubated in 0.04 *M* Tris, pH 7.5, for 10 minutes at 30° with varying concentrations of analogues prior to the addition of Pyr5-P (1.6×10^{-4} *M*). After an additional 10 minutes incubation, aliquots were diluted in EDTA-glycerophosphate, pH 6.7, for immediate enzymatic assay.

nase systems from *Streptococcus faecalis.*[14] Sandman and Snell found[15] that ω-methylpyridoxal-5-phosphate had about 10 per cent of the activity of pyridoxal-5-phosphate in activating liver aspartic-glutamic transaminase. Table 3 shows that apophosphorylase *a* can be activated to the extent of at least 50 per cent by ω-methylpyridoxal-5-phosphate.[16] The data from experiments in which pyridoxal-5-phosphate and its analogue were added simultaneously show that the apoenzyme has a somewhat greater affinity for the former substance. The fact that neither pyridoxal nor 5-deoxypyridoxal reactivates the apoenzyme, while pyridoxal-5-phosphate and ω-methylpyridoxal-5-phosphate are effective in returning enzyme activity, shows that the hydroxymethylphosphate group at position 5 of the pyridine ring is required in the constitution of the active center in phosphorylase. However, as mentioned above, all efforts to demonstrate the direct participation of this phosphate group in the phosphorylase reaction have failed.

Resolution of Phosphorylase b.—Phosphorylase *b* was prepared by the method of Fischer and Krebs[17] and crystallized in the presence of AMP-5′, Mg^{++}, and cysteine. The third crystals were dissolved in water, dialyzed overnight, treated with

TABLE 3

ACTIVATION OF APOPHOSPHORYLASE *a* BY PYRIDOXAL-5-PHOSPHATE AND ω-METHYLPYRIDOXAL-5-PHOSPHATE

SUBSTANCES INCUBATED* SIMULTANEOUSLY WITH 1×10^{-6} *M* APOENZYME		
PYR-P ($M \times 10^6$)	ω-CH_3-PYR-P ($M \times 10^6$)	ACTIVITY (UNITS/MG PROTEIN)
None	None	250
10.4	None	1,900
10.4	11	1,600
10.4	25	1,075
24	11	1,885
None	11	847
None	37	970

* Incubation in 0.027 *M* Tris, pH 7.5, for 15 minutes at 30° prior to dilution in 0.02 *M* glycerophosphate, pH 6.7, and assay for phosphorylase activity in the presence of AMP-5′.

Norit A, and the solution kept at 5° under toluene. It was possible to resolve phosphorylase *b* in the presence of freshly prepared 0.06 *M* cysteine with 0.08 *M* Na citrate buffer at pH 3.5, provided that the time of treatment was exactly 1 minute at 0°. The apoenzyme was then precipitated by the addition of $(NH_4)_2SO_4$, as in the case of apophosphorylase *a*, and the protein washed with cold, 0.5 saturated $(NH_4)_2SO_4$, pH 8.5.[7] The washed apoenzyme was dissolved in cysteine-glycerophosphate buffer, pH 7.0.

In general, apophosphorylase *b* prepared by this method and the protein after recombination with pyridoxal-5-phosphate were rather unstable as compared with unresolved phosphorylase *b* or even with apophosphorylase *a*, and, accordingly, attempts were made to remove pyridoxal-5-phosphate under milder conditions. It was found that incubation of phosphorylase *b* with 0.1 *M* Na glycerophosphate–0.08 *M* cysteine, pH 6.0, at 0° for 12 hours resulted in the complete resolution of the enzyme. In contrast, incubation of phosphorylase *a* under these same conditions for as long as 18 hours was without effect on this enzyme. The rate of pyridoxal-5-phosphate removal from phosphorylase *b* was pH-dependent. Thus, the time required to release 90 per cent of the total amount at 0° was 3 hours at pH 5.4 and 10 hours at pH 6.0. No pyridoxal-5-phosphate was released after even 18 hours of exposure to glycerophosphate-cysteine at pH 6.5. Incubation of phosphorylase *b* for 4 hours in Na glycerophosphate, pH 5.4, in the absence of cysteine did not result in any measurable release of pyridoxal-5-phosphate.

After treatment of the enzyme at pH 6.0 with cysteine as described above, the protein was precipitated at 66 per cent saturation in $(NH_4)_2SO_4$ by the addition of pH 8.0, saturated $(NH_4)_2SO_4$.[7] The protein was washed with 0.5 saturated $(NH_4)_2SO_4$, pH 7, and then dissolved in either 0.04 *M* Na glycerophosphate, pH 7.0, or 0.03 *M* cysteine–0.04 *M* Na glycerophosphate, pH 7.0, and dialyzed for 1 hour in the cold against a similar solution.

Ultracentrifugal Properties of Apophosphorylase b.—The apoenzyme in cysteine-glycerophosphate, prepared by the second method described above and having a residual activity of 116 units/mg protein, showed one broad peak, $s_{20}' = 7.6$, in the ultracentrifuge. After recombination with pyridoxal-5-phosphate, the activity had risen to 1,160 units/mg protein and the peak, $s_{20}' = 7.8$, became distinctly sharper. A minor shoulder, $s_{20}' = ca.\ 12$, appeared in the pattern of unresolved phosphorylase *b*, as well as in some preparations of the apoenzyme. These observa-

tions indicate that apophosphorylase *b* exists mostly as units with the molecular weight of phosphorylase *b*. There appears to be much less of the monomer unit present in solutions of apophosphorylase *b* than is the case with apophosphorylase *a*, which has been discussed above. In both cases, however, incubation with pyridoxal-5-phosphate reduced the heterogeneity of the apoenzyme preparation and restored the ultracentrifugal pattern characteristic of the unresolved enzyme.

Enzymatic Conversion of Apophosphorylase b to Apophosphorylase a.—The possibility of converting apophosphorylase *b* to apophosphorylase *a* was investigated, using the ATP-dependent kinase preparation from rabbit muscle, described by Krebs *et al.*[18] After incubating apophosphorylase *b* containing about 25 per cent of unresolved enzyme with the phosphorylase kinase at pH 7.7 for 40 minutes at 25°, aliquots were diluted 1:200 with cysteine-glycerophosphate, pH 6.8, and the enzymatic activity in the presence of AMP-5′ (due to phosphorylase *a* + phosphorylase *b*) and in the absence of AMP-5′ (due to phosphorylase *a*) was determined before and after preincubation with pyridoxal-5-phosphate. A control tube to which no converting enzyme had been added was treated similarly. Table 4 shows

TABLE 4

ENZYMATIC CONVERSION OF APOPHOSPHORYLASE *b* TO APOPHOSPHORYLASE *a*

	ACTIVITY (UNITS/MG PROTEIN)			
	Without Pyr-P		With Pyr-P	
	(− AMP-5′)	(+ AMP-5′)	(− AMP-5′)	(+ AMP-5′)
− Phosphorylase kinase	0	360	0	1,210
+ Phosphorylase kinase	310	390	1,170	1,500

that the phosphorylase kinase had converted the residual phosphorylase *b* to phosphorylase *a* as revealed by the appearance of activity without AMP-5′ in the absence of pyridoxal-5-phosphate. Of particular interest was that, in addition, apophosphorylase *b* had been transformed to apophosphorylase *a*, which responded maximally to pyridoxal-5-phosphate. This was shown by the appearance of 1,170 units/mg protein activity in the absence of AMP-5′ after kinase action. Ultracentrifugal examination of the control showed the usual pattern of apophosphorylase *b* in which most of the protein was present in a very broad peak, $s_{20}' = 7.7$. The reaction mixture, which had been incubated with phosphorylase kinase, had a single component, $s_{20}' = 12.0$, corresponding to apophosphorylase *a*. These findings demonstrate that the action of phosphorylase kinase does not depend upon the presence of pyridoxal-5-phosphate at the enzymatically active site of phosphorylase. Fischer *et al.*[19] have recently reported that they were able to change the nature of the linkage of pyridoxal-5-phosphate to phosphorylase *b* by treatment with $NaBH_4$ and that this modified protein could be converted by phosphorylase kinase to a protein having the enzymatic activity of phosphorylase *a*.

Reaction of Pyridoxal-5-Phosphate with Apophosphorylase b.—Apophosphorylase *b* was prepared by incubation with cysteine as described above, followed by dialysis against Na glycerophosphate buffer, pH 7.0 at 0°. This preparation showed only a slight shoulder of absorption in the region of 330 mμ, where, as reported by Kent *et al.*,[12] phosphorylase *b* has a distinct, though small, absorption maximum. When 2 moles of pyridoxal-5-phosphate per mole of protein were added to the solution of the apoenzyme at pH 7.0, the absorption of the mixture at 330 mμ rose. At 7°,

about 60 minutes were required for complete reaction of pyridoxal-5-phosphate with the protein to occur, as judged by the attainment of a maximum absorption value at 330 mμ and the complete disappearance of any absorption at 390 mμ due to free pyridoxal-5-phosphate. Figure 2 shows an experiment in which the apoenzyme was titrated with various amounts of pyridoxal-5-phosphate in Na glycerophosphate, pH 7.0, at room temperature. The course of combination of the reactants was followed as a decrease in absorption at 390 mμ. Observations at various times showed that equilibrium was attained by 30 minutes after mixing. A sharp break in the curve occurred when 1.9 moles of pyridoxal-5-phosphate had been added. The enzymatic activity of the recombined enzyme was measured after 2 hours, and, in Figure 2, the per cent reactivation observed is plotted as a function of the amount of pyridoxal-5-phosphate added. It was found that the enzymatic activity was constant after 1.9 moles had reacted. These data substantiate earlier findings[2] from this laboratory that phosphorylase *b* has 2 moles of pyridoxal-5-phosphate bound to it. Kent *et al.*[12] have reported that phosphorylase *b* contains about 2.5 moles of pyridoxal-5-phosphate per mole of protein.

The kinetics of the recombination of pyridoxal-5-phosphate with apophosphorylase *b* were investigated in 0.04 *M* Na glycerophosphate–0.005 *M* EDTA, pH 7.0, at 25°. Exactly 2 moles of pyridoxal-5-phosphate were added initially per mole of protein present. Hence, if the two sites of reaction of pyridoxal-5-phosphate with phosphorylase *b* are regarded as equivalent and independent, the concentrations of the two reactants were initially equal. A Beckman DU spectrophotometer equipped with a Brown recorder was used to measure the decrease in absorption at 390 mμ. A minimum value was obtained 15 minutes after mixing. This reading (0.060) was assumed to be the absorption of fully recombined phosphorylase *b* at 390 mμ; from it a correction was calculated for the contribution to the observed extinction of the amount of recombined phosphorylase *b* which was present at each time during the reaction. Each of these calculated extinction values was subtracted from the corresponding observed readings to give the optical density of free pyridoxal-5-phosphate shown in the third column of Table 5. On the assumption that the reaction was bimolecular and that the reacting species were present at equal concentrations, as discussed above, the constant, k, in the equation: $kt = 1/C_t - 1/C_0$, was calculated for each time period after the first. Since the absolute time after mixing at which the first reading was taken was not known accurately, zero time was set equal to the reading at 10 seconds. The observed molar extinction (3,760) for pyridoxal-5-phosphate in the buffer used in the experiment was used in

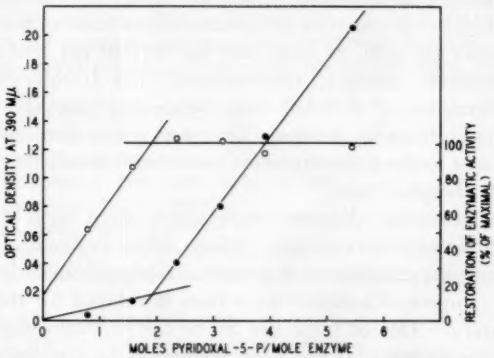


FIG. 2.—Titration of the reaction of pyridoxal-5-phosphate with apophosphorylase *b*. ○, restoration of enzymatic activity; ●, optical density of free pyridoxal-5-phosphate.

TABLE 5

KINETICS OF COMBINATION OF PYRIDOXAL-5-PHOSPHATE WITH APOPHOSPHORYLASE *b**

Time After Mixing (Seconds)	Observed Optical Density at 390 mμ	Calculated Optical Density at 390 mμ Due to Free Pyr-P	*k* (Liters/Mole/Sec)
0	0.380	0.380	....
10†	0.320	0.309	....
16.6	0.300	0.285	156
23.2	0.280	0.261	170
29.6	0.260	0.238	185
39.6	0.240	0.214	182
51.0	0.220	0.190	186
66.0	0.200	0.166	187
88.0	0.180	0.142	184
117.0	0.160	0.119	181
163.0	0.140	0.095	179
228.0	0.120	0.071	187
900	0.060	0.000†	

* The concentration of the apoenzyme was 5×10^{-6} *M*.
† See text.

converting extinction values to molar concentrations. The data of Table 5 show that the reaction of pyridoxal-5-phosphate with apophosphorylase *b* is a second-order reaction, at least over the first 80 per cent of its course. The rather large reaction constant, approximately 185 l/mole/sec, presumably pertains to the formation of a Schiff base between pyridoxal-5-phosphate and the protein (ϵ-lysine[19]) amino groups. This step is rate-limiting and is followed by a rearrangement to the stable structure that characterizes the linkage of pyridoxal-5-phosphate in phosphorylase.

Summary.—Various experiments have been done on the mechanism of the phosphorylase reaction. These failed to demonstrate any direct participation of the phosphate group of pyridoxal-5-phosphate in the enzymatic reaction.

Improved methods have been developed for the resolution of phosphorylases *a* and *b*. One of these has led to the crystallization of apophosphorylase *a*. Apophosphorylase *b* has been converted to apophosphorylase *a* with phosphorylase kinase and adenosine triphosphate.

The apophosphorylases exist in solution as a mixture of molecular species corresponding in size to a basic monomer unit (mol. wt. *ca.* 125,000) and to multiples of it. Recombination with pyridoxal-5-phosphate, pyridoxal, or 5-deoxypyridoxal causes the protein to resume its original molecular size. The latter two analogues do not restore enzymatic activity. On the other hand, ω-methylpyridoxal-5-phosphate can reactivate to the extent of at least 50 per cent. That pyridoxal and 5-deoxypyridoxal combine with apophosphorylase *a* is shown by the fact that preincubation with these substances inhibits subsequent reaction of the protein with pyridoxal-5-phosphate. Combination of the apoenzyme with pyridoxal-5-phosphate can be prevented by preincubation of the latter compound with the carbonyl reagent, isonicotinic acid hydrazide. However, no inhibition of enzymatic activity is observed when phosphorylase *a* is incubated with the carbonyl reagent. These findings prove that, although the combination of apophosphorylase *a* with pyridoxal-5-phosphate is through the 4-formyl group of the latter, no Schiff-base linkage exists in phosphorylase.

When investigated spectrophotometrically, the combination of apophosphorylase

b with 2 moles of pyridoxal-5-phosphate exhibits the kinetics of a second-order reaction. It is concluded that the formation of a Schiff base between pyridoxal-5-phosphate and protein amino groups is the rate-limiting, intermediate reaction and is followed by further rearrangement to the stable bond structure in phosphorylase.

* This work has been supported in part by a research grant from the Nutrition Foundation.

† Fellow of the Netherlands Organization for Pure Research (Z.W.O.).

[1] T. Baranowski, B. Illingworth, D. H. Brown, and C. F. Cori, *Biochim. et Biophys. Acta*, **25**, 16, 1957.

[2] C. F. Cori and B. Illingworth, these PROCEEDINGS, **43**, 547, 1957.

[3] M. Cohn and G. T. Cori, *J. Biol. Chem.*, **175**, 89, 1948.

[4] The following abbreviations are used: G_1-P,α-glucose-1-phosphate; P_{inorg}, inorganic orthophosphate; Pyr_5-P, pyridoxal-5-phosphate; Tris, tris-(hydroxymethyl)-amino methane; EDTA, ethylenediaminetetraacetic acid; AMP-5′,5′-adenylic acid; ATP, adenosine triphosphate.

[5] The authors are grateful to Dr. Luis Glaser for preparing this compound enzymatically from uniformly labeled C^{14}-starch.

[6] C. F. Cori, G. T. Cori, and A. A. Green, *J. Biol. Chem.*, **151**, 39, 1943.

[7] $(NH_4)_2SO_4$ saturated at 5°. The pH of the solution was adjusted with NH_3 and determined with the Beckman pH meter set at 10°, after dilution of the cold solution to 0.25 saturation.

[8] Na thioglycolate was selected because apophosphorylase *a* could be readily dissolved at room temperature in solutions of this sulfhydryl compound and repeatedly crystallized in the cold. Usually, crystallization of the apoenzyme did not occur when cysteine-glycerophosphate was substituted for thioglycolate.

[9] N. B. Madsen and C. F. Cori, *J. Biol. Chem.*, **223**, 1055, 1956.

[10] The authors are indebted to Dr. Karl Folkers for a generous sample of this substance.

[11] Kindly supplied by Dr. J. O. Lampen, Squibb Institute for Medical Research.

[12] A. B. Kent, E. G. Krebs, and E. H. Fischer, *J. Biol. Chem.*, **232**, 549, 1958.

[13] M. Ikawa and E. E. Snell, *J. Am. Chem. Soc.*, **76**, 637, 1954.

[14] J. Olivard and E. E. Snell, *J. Biol. Chem.*, **213**, 215, 1955.

[15] R. Sandman and E. E. Snell, *Proc. Soc. Exptl. Biol. Med.*, **90**, 63, 1955.

[16] The authors are indebted to Dr. E. E. Snell for making a sample of this substance available to them.

[17] E. H. Fischer and E. G. Krebs, *J. Biol. Chem.*, **231**, 65, 1958.

[18] E. G. Krebs, A. B. Kent, and E. H. Fischer, *J. Biol. Chem.*, **231**, 73, 1958.

[19] E. H. Fischer, A. B. Kent, E. R. Snyder, and E. G. Krebs, *J. Am. Chem. Soc.*, **80**, 2906, 1958.

ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID. V. CHEMICAL COMPOSITION OF ENZYMATICALLY SYNTHESIZED DEOXYRIBONUCLEIC ACID

BY I. R. LEHMAN, STEVEN B. ZIMMERMAN,* JULIUS ADLER,† MAURICE J. BESSMAN,‡ E. S. SIMMS, AND ARTHUR KORNBERG

DEPARTMENT OF MICROBIOLOGY, WASHINGTON UNIVERSITY SCHOOL OF MEDICINE, ST. LOUIS, MISSOURI

Communicated October 16, 1958

The enzymatic synthesis of DNA[1] in sufficient excess over the DNA used to prime the reaction[2, 3] has enabled us to examine the chemical composition of the synthetic product. As has already been demonstrated, this DNA consists of deoxynucleotides linked by typical 3′-5′ phosphodiester bonds, and all four of the deoxy-

nucleotides which occur naturally in DNA are present and required for its synthesis.[3] The purpose of this investigation was to answer two questions: (1) Does the synthesized DNA show the equivalence of purines to pyrimidines characteristic of DNA? and (2) Does the base composition of the DNA primer influence the composition of the synthesized product?

The results to be presented answer both questions in the affirmative and further suggest that the added DNA from the very outset of the reaction is serving as a template for the synthesis of new DNA.

MATERIALS AND METHODS

Materials.—Deoxynucleoside triphosphates were synthesized by the method of Smith and Khorana.[4] Uniformly labeled C^{14}-deoxynucleoside triphosphates were prepared as described previously.[5] The DNA-synthesizing enzyme was the "polymerase" fraction VII described elsewhere.[2] DNA of *Escherichia coli* and *Aerobacter aerogenes* was extracted from fresh or frozen cells lysed with sodium dodecyl sulfate.[6] Precipitation of the DNA by adding two volumes of cold absolute ethanol, followed by solution in cold 0.14 *M* NaCl (containing 0.01 *M* sodium citrate) and then further deproteinization with sodium dodecyl sulfate,[7] was carried out once or twice. Then the DNA was treated with ribonuclease (crystalline, Worthington Biochemical Corp. product, pretested for absence of deoxyribonuclease activity) and Norit to remove contaminating ribonucleic acid. After precipitating again with cold ethanol and dissolving as above, preparations were obtained which had reduced viscosities of 57 and 87 deciliters/gm for *E. coli* and *A. aerogenes* samples, respectively, and gave absorbancy values (α *M* $\times$ 10^{-3}) at 260 mμ of 6.4–6.9 based on deoxyribose. Protein contamination, as measured by the method of Lowry *et al.*[8] was below 2 per cent; the over-all yield was over 80 per cent, measured by the Dische reaction.[9] The DNA of *Mycobacterium phlei* was isolated by the same procedure, except that cell lysis was obtained by alumina grinding. While the *coli* and *aerogenes* products formed long white fibers on ethanol precipitation and could be quantitatively pulled out on rods, the *phlei* material had to be recovered by centrifuging. The absorbancy was 6.5×10^3 based on deoxyribose, protein content was about 5 per cent, and the reduced viscosity 24 deciliters/gm. Bacteriophage DNA was prepared from purified T_2 bacteriophage by osmotic shock,[10] followed by removal of the intact bacteriophage and ghosts by centrifugation at 140,000 $\times$ *g*. for 1 hour. Calf thymus DNA was isolated by the method of Kay, Simmons, and Dounce.[7]

Methods.—The enzymatic synthesis of DNA and the isolation of the synthesized material were achieved as follows: The incubation mixtures (3.0 ml.) contained 1.0 μmole each of dCTP, dATP, dTTP, and dGTP, 200 μmoles of potassium phosphate buffer (*p*H 7.4), 20 μmoles of $MgCl_2$, 36 μg. of "polymerase" fraction VII, and 100μg. of the appropriate DNA primer. After incubation for 240 minutes at 37°, the mixtures were treated with 4 *M* NaCl to give a final concentration of 0.2 *M* and then heated for 5 minutes at 70°. The mixtures were dialyzed against several changes of 0.2 *M* NaCl to remove unreacted deoxynucleoside triphosphates, followed by dialysis against distilled water to remove the NaCl. They were concentrated to a small volume and then hydrolyzed with formic acid. The base composition of the various primers and products was determined by the method of Wyatt and Cohen.[11]

The recoveries of bases from the paper chromatograms ranged from 96 to 101 per cent.

The incorporation of C^{14}-labeled deoxynucleoside triphosphates into DNA was measured as reported previously.[2]

RESULTS

The Base Composition of Enzymatically Synthesized DNA Resembles That of Its Primer.—In each enzymatically synthesized DNA sample the content of adenine approximated the amount of thymine, and the guanine content was about the same as the cytosine, so that the total amount of purine in each sample was the same as the total amount of pyrimidine (i. e., A = T; G = C; A + G = T + C) (Table 1).

TABLE 1*

PURINE AND PYRIMIDINE COMPOSITION OF ENZYMATICALLY SYNTHESIZED DNA

DNA		No. of Analyses	A	T	G	C	$\frac{A+T}{G+C}$	$\frac{A+G}{T+C}$
M. phlei	primer	3	0.65	0.66	1.35	1.34	0.49 (0.48–0.49)	1.01 (0.98–1.04)
	product	3	0.66	0.80	1.17	1.34	0.59 (0.57–0.63)	0.85 (0.78–0.88)
A. aerogenes	primer	1	0.90	0.90	1.10	1.10	0.82	1.00
	product	3	1.02	1.00	0.97	1.01	1.03 (0.96–1.13)	0.99 (0.95–1.01)
E. coli	primer	2	1.00	0.97	0.98	1.05	0.97 (0.96–0.99)	0.98 (0.97–0.99)
	product	2	1.04	1.00	0.97	0.98	1.02 (0.96–1.07)	1.01 (0.96–1.06)
Calf thymus	primer	2	1.14	1.05	0.90	0.85	1.25 (1.24–1.26)	1.05 (1.03–1.08)
	product	6	1.19	1.19	0.81	0.83	1.46 (1.22–1.67)	0.99 (0.82–1.04)
T_2 bacteriophage	primer	2	1.31	1.32	0.67	0.70	1.92 (1.86–1.97)	0.98 (0.95–1.01)
	product	2	1.33	1.29	0.69	0.70	1.90 (1.82–1.98)	1.02 (1.01–1.03)
Synthetic A-T copolymer†		1	1.99	1.93	<0.05	<0.05	>40	1.05

* A, T, G, and C refer, respectively, to adenine, thymine, guanine, and cytosine except that C in the case of T^2 bacteriophage primer refers to hydroxymethylcytosine. The values given represent averages of the number of analyses indicated. The figures in parentheses represent the range of values obtained.

† A copolymer of deoxyadenylate and thymidylate is formed by the "polymerase" system *in the absence of added DNA* after a lag period of 3–6 hours. Once formed and isolated, this A-T polymer initiates the synthesis of new polymer without any time lag and, despite the presence of all four deoxynucleoside triphosphates in the incubation mixture, contains exclusively A and T. These and further details will be reported at a future date by H. K. Schachman and the authors.

Furthermore, the ratio A + T/G + C in the enzymatic product was always close to that in the corresponding primer; primer values ranged from 0.49 for *M. phlei* to greater than 40 for an enzymatically synthesized copolymer of deoxyadenylate and thymidylate. It should be pointed out that the net amount of DNA synthesized in each case was at least ten times the primer DNA added, except in the *M. phlei* experiments, where it was only fourfold. Thus in all but the *M. phlei* samples, over 90 per cent of the bases were derived from the nucleotide substrates for the reaction.

The Composition of the Product Is Established Early in the Reaction.—The base ratios of the synthesized product were essentially the same, regardless of whether it was isolated early in the reaction or after synthesis had stopped. Thus with calf thymus DNA as primer, the A + T/G + C was found to be 1.34 after a threefold net increase in DNA and 1.42 after net synthesis had exceeded eight fold.

To determine whether this result held even at the earliest stages of reaction, isotopically marked substrates were used to distinguish the newly synthesized DNA. In such an experiment, two identical reaction mixtures were set up as described under "Methods," one containing C^{14}-dCTP and the other C^{14}-dTTP. The amount of enzyme and length of incubation period were adjusted to permit an increase of

DNA as low as 2 per cent relative to the DNA added as primer. Isotope incorporation into DNA was determined as described previously for the assay of "polymerase."[2] The T/C ratio of the product was found to be similar to that of the primer when the net increases in DNA ranged from 2 to 500 per cent (Table 2).

TABLE 2*

EFFECT OF EXTENT OF NET SYNTHESIS ON COMPOSITION OF ENZYMATICALLY SYNTHESIZED DNA

Primer	Exp. No.	Time (Hours)	Increase in DNA (Per Cent)	T Incorporated (mμMoles)	C Incorporated (mμMoles)	T/C
M. phlei DNA primer						0.49
M. phlei DNA product	1		2	0.047	0.11	0.42
	2	1	35	0.97	2.05	0.47
		2	86	2.13	5.03	0.43
		4	163	4.20	9.54	0.44
		6	...	10.25	9.88	1.04
		7	...	24.20	12.10	2.00
Calf thymus DNA primer						1.24
Calf thymus DNA product	3		8	0.40	0.28	1.43
	4	0.5	63	3.08	2.43	1.27
		1	197	9.10	7.72	1.20
		3	534	31.60	20.60	1.53
A. aerogenes DNA primer						0.82
A. aerogenes DNA product	5	0.5	18	0.74	0.93	0.79
		1.0	56	2.59	2.89	0.91
		3.0	344	15.00	17.75	0.84
		4.0	406	16.62	21.00	0.80

* Duplicate reaction mixtures were used as described under "Methods," with the exception that C^{14} dTTP was used in one vessel and C^{14} dCTP was used in the second. Aliquots were removed at the intervals indicated and assayed for the incorporation of isotope into DNA.

It may be noted in Table 1 that the value for the ratio A + T/G + C was slightly higher than that for the primer in some cases; lower ratios in the product have never been observed. It seems most probable that these divergencies between primer and product values are due to contamination with the deoxyadenylate-thymidylate (A-T) copolymer which is produced after 3–6-hour lag periods (see n.† to Table 1). For example, when DNA synthesis with *M. phlei* DNA as primer was examined over an extended reaction time interval (Table 2), the T/C ratio of the product remained the same as that of the primer for 4 hours, after which time the synthesis of the A-T copolymer seemed to predominate.

Distortion of the Relative Concentrations of the Deoxynucleoside Triphosphates Does Not Affect the Base Ratio of the Enzymatic Product.—While the results in Tables 1 and 2 were obtained with equimolar concentrations of the deoxynucleoside triphosphates in the synthetic reaction, similar results were also observed with markedly distorted substrate concentrations (Table 3). The starting concentration of dTTP

TABLE 3*

EFFECT OF RELATIVE SUBSTRATE CONCENTRATIONS ON COMPOSITION OF SYNTHETIC DNA

Substrates (Relative Molar Concentration)				Net Synthesis	Products	
dCTP	dGTP	dTTP	dATP		A + T / G + C	A + G / C + T
1.0	1.0	1.0	1.0	11x	1.98	1.00
1.0	1.0	0.2	1.0	6x	1.82	1.04
1.0	1.0	0.2	0.2	6x	1.82	0.97
T_2 bacteriophage DNA as primer					1.92	0.98

* The reaction mixtures were the same as described under "Methods," except that 1.5 or 0.3 μmole of the substrates were used as noted above and 50 μg. of T_2 bacteriophage DNA was used as primer.

or of both dTTP and dATP was lowered to one-fifth the level of the other triphosphates. As the reaction proceeded, the disparity in relative concentrations was exaggerated, and presumably the extent of DNA synthesis was curtailed by the exhaustion of the limiting substrate. Nevertheless, the product synthesized maintained the same A + T/G + C ratio as the primer, and the purine content was equivalent to the pyrimidine content.

DISCUSSION

A close correspondence of adenine to thymine and of guanine to cytosine has been established as characteristic of DNA isolated from all natural sources[12, 13] and is a key element in the Watson and Crick structure for DNA.[14] Such equivalence of purines to pyrimidines was observed in our synthesized DNA, regardless of the relative concentrations of the substrates present in the reaction mixture or of the extent of the reaction. From these findings, as well as earlier observations on the replacement of the naturally occuring deoxynucleotide substrates by related analogues,[15] it is clear that the relationship of adenine to thymine and of guanine to cytosine is an inherent feature of DNA synthesis by the "polymerase" enzyme of *E. coli*. However, these results alone do not implicate the added DNA as a template in the process, nor do they explain why all four of the deoxynucleotides are essential for a significant amount of DNA synthesis.

A crucial and sensitive test of the function of the added DNA in the reaction was the use of a variety of DNA samples which differ widely in their content of adenine and thymine relative to guanine and cytosine. Here the nice agreement found between the base composition of the added DNA and the synthesized DNA is decisive evidence for the participation of the added DNA in the reaction; a most plausible interpretation is that the primer DNA provides a template. There are implications of the template hypothesis that can and should be tested. One consequence of replication would be the reproduction of the transforming activity inherent in DNA samples from *Hemophilus influenzae*[16] and *Diplococcus pneumoniae*.[17] In preliminary experiments in which *H. influenzae* or *D. pneumoniae* DNA with the marker for streptomycin resistance was used as primer, there was no increase in transforming activity despite chemically determined increases of six- to eightfold in DNA content. Such experiments have, however, been complicated by the persistence of sufficient deoxyribonuclease activity in the most purified "polymerase" preparations to cause destruction of 30–90 per cent of the transforming activity added initially. More definitive experiments must therefore await an enzyme preparation in which such DNase activity has been completely eliminated. Another result of replication of DNA chains would be the influence of the molecular size of the template on the size of the product. Earlier studies[18] have, in fact, shown that the molecular size of the product synthesized with calf thymus DNA as primer is very close to that of its primer. Similar experiments should also be attempted with a monodisperse primer of smaller size.

It should be pointed out that T_2 bacteriophage DNA, which contains hydroxymethylcytosine, supports extensive DNA synthesis, although this base is not provided as a substrate. However, the base required for pairing with the hydroxymethylcytosine of the phage DNA is guanine, and the guanine may then pair with the cytosine that is provided in the enzymatic reaction.

SUMMARY

Analysis of the purine and pyrimidine composition of DNA, enzymatically synthesized in the presence of a variety of DNA primers, reveals that the content of adenine residues is equal to that of thymine and that the number of guanine residues equals the cytosine residues. These results agree with the composition data for DNA samples isolated from nature and with the requirements of the DNA structure proposed by Watson and Crick.

The ratio of the number of adenine-thymine pairs to the number of guanine-cytosine pairs in an enzymatically synthesized sample reflects the ratio present in the primer used in its synthesis; the ratios in the primers tested varied from 0.5 to >40.

Distortion of the relative concentrations of the deoxynucleotide substrates in the reaction mixture or variation of the extent of enzymatic synthesis from 2 to more than 1,000 per cent had no effect on the base composition of the DNA product.

These results suggest that the enzymatic synthesis of DNA by the "polymerase" of *E. coli* represents the replication of a DNA template.

This investigation was supported by research grants from the National Institutes of Health of the Public Health Service and the National Science Foundation. We are grateful to S. R. Kornberg for the isolation of the deoxyribonucleic acids used in this investigation.

* National Science Foundation Predoctoral Fellow.

† Life Insurance Medical Research Fund Postdoctoral Fellow.

‡ U.S. Public Health Service Postdoctoral Fellow. Present address, McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland.

[1] The abbreviations used in this report are dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate; dTTP, thymidine triphosphate; DNA, deoxyribonucleic acid.

[2] I. R. Lehman, M. J. Bessman, E. S. Simms, and A. Kornberg, *J. Biol. Chem.*, **233**, 163, 1958.

[3] M. J. Bessman, I. R. Lehman, E. S. Simms, and A. Kornberg, *J. Biol. Chem.*, **233**, 171, 1958.

[4] M. Smith and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 1141, 1958.

[5] J. Adler, I. R. Lehman, M. J. Bessman, E. S. Simms, and A. Kornberg, these PROCEEDINGS, **44**, 641, 1958.

[6] S. Zamenhof, B. Reiner, R. de Giovanni, and K. Rich, *J. Biol. Chem.*, **219**, 165, 1956.

[7] E. R. M. Kay, N. S. Simmons, and A. L. Dounce, *J. Am. Chem. Soc.*, **74**, 1724, 1952.

[8] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265, 1951.

[9] Z. Dische, in *The Nucleic Acids*, ed. E. Chargaff and J. N. Davidson (New York: Academic Press, 1955), **1**, 285.

[10] R. M. Herriott, *J. Bact.*, **61**, 752, 1951.

[11] G. R. Wyatt and S. S. Cohen, *Biochem. J.*, **55**, 774, 1953.

[12] E. Chargaff, *Federation Proc.*, **10**, 654, 1951.

[13] G. R. Wyatt, in *The Chemistry and Physiology of the Nucleus* (New York: Academic Press, 1952), p. 201.

[14] J. D. Watson and F. H. C. Crick, *Nature*, **171**, 737, 1953.

[15] M. J. Bessman, I. R. Lehman, J. Adler, S. B. Zimmerman, E. S. Simms, and A. Kornberg, these PROCEEDINGS, **44**, 633, 1958.

[16] H. E. Alexander and G. Leidy, *J. Exptl. Med.*, **97**, 17, 1953.

[17] R. D. Hotchkiss, *Cold Spring Harbor Symposia Quant. Biol.*, **16**, 457, 1951.

[18] H. K. Schachman, I. R. Lehman, M. J. Bessman, J. Adler, E. S. Simms, and A. Kornberg, *Federation Proc.*, **17**, 304, 1958.

*ELECTRON-DONOR AND -ACCEPTOR PROPERTIES OF BIOLOGICALLY IMPORTANT PURINES, PYRIMIDINES, PTERIDINES, FLAVINS, AND AROMATIC AMINO ACIDS**

By Bernard Pullman and Alberte Pullman†

INSTITUTE FOR MUSCLE RESEARCH, MARINE BIOLOGICAL LABORATORY, WOODS HOLE, MASSACHUSETTS

Communicated by Albert Szent-Györgyi, September 24, 1958

INTRODUCTION

The recent discovery by Isenberg and Szent-Györgyi[1] of the formation, at neutral pH, of a tryptophan-riboflavin complex in which a riboflavin molecule has taken up one electron from the tryptophan and the possible important biological implications of this phenomenon draw attention to the general problem of electron-donor and -acceptor properties of biologically important substances like purines, pyrimidines, pteridines, etc. As no experimental data seem to be available about the molecular ionization potentials or electroaffinites of these molecules, we have used the results of quantum-mechanical calculations of the distribution of electronic energy levels[2] to obtain the corresponding information.

The calculations have been carried out by the molecular-orbital method which yields the energies of the molecular orbitals of the mobile or π electrons of the system in the form $E_i = \alpha + K_i\beta$, where α is the coulomb integral and β is the resonance integral of the method.[3] Positive values of K_i correspond to occupied (bonding) orbitals, negative values of K_i to empty (antibonding) orbitals. The smallest positive value of K_i corresponds to the highest occupied molecular orbital (h.o.m.o.), and the comparison of the value of this parameter in a series of related compounds gives the relative value of their ionization potentials: the smaller the value, the lower the ionization potential and consequently the greater the electron donor capacity of the molecule. The smallest negative value of K_i corresponds to the lowest empty molecular orbital (l.e.m.o.), and the comparison of the value of this parameter in a series of related compounds measures the relative value of their electroaffinity: the smaller the value, the greater the electroaffinity.

THE RESULTS

Table 1 gives the results of the calculations for the biologically fundamental purines, pyrimidines, and aromatic amino acids, as well as for riboflavin and for a number of pteridines.

DISCUSSION

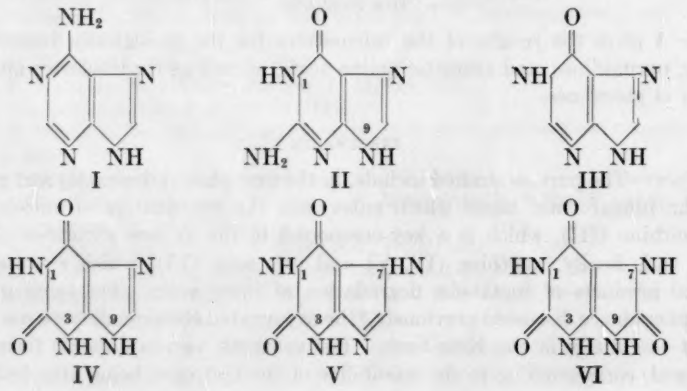
Purines.—The purines studied include, in the first place, adenine (I) and guanine (II), the fundamental bases which enter into the constitution of nucleic acids; hypoxanthine (III), which is a key-compound in the *de novo* synthesis of these acids; and, finally, xanthine (IV, V) and uric acid (VI), which represent the essential products of metabolic degradation of these acids. Following physicochemical evidence discussed previously,[2] the oxygenated compounds were considered to exist essentially in the Keto form. For xanthine, two tautomeric forms were considered, corresponding to the possibility of the hydrogen being attached to N_7

TABLE 1

Compound	Energy of Highest Occupied Molecular Orbital	Energy of Lowest Empty Molecular Orbital
Adenine	0.486	−0.865
Guanine	0.307	−1.050
Hypoxanthine	0.402	−0.882
Xanthine (IV)	0.397	−1.197
Xanthine (V)	0.442	−1.005
Uric acid	0.172	−1.194
1-methyl guanine	0.303	−1.064
9-methyl guanine	0.302	−1.074
1-methyl Xanthine (IV)	0.397	−1.198
3-methyl Xanthine (IV)	0.354	−1.197
9-methyl Xanthine (IV)	0.394	−1.213
1-methyl Xanthine (V)	0.442	−1.009
3-methyl Xanthine (V)	0.395	−1.009
7-methyl Xanthine (V)	0.429	−1.041
1-methyl Uric acid	0.172	−1.201
3-methyl Uric acid	0.153	−1.204
7-methyl Uric acid	0.133	−1.200
9-methyl Uric acid	0.161	−1.204
Uracil	0.597	−0.960
Thymine	0.510	−0.958
Cytosine	0.595	−0.795
5-methylcytosine	0.530	−0.796
Barbituric acid	1.033	−1.295
Alloxane	1.033	−0.757
Phenylallanine*	0.908	−0.993
Tyrosine†	0.792	−1.000
Histidine‡	0.660	−1.160
Tryptophan§	0.534	−0.863
Riboflavin‖	0.500	−0.344
Pteridine	0.864	−0.386
2-amino-4-hydroxypteridine	0.489	−0.650
2,4-diaminopteridine	0.544	−0.508
2,4-dihydroxypteridine	0.653	−0.663
Folic acid	0.526	−0.647

* The π electron system of this molecule is assumed to be that of toluene.
† The π electron system of this molecule is assumed to be that of phenol.
‡ The π electron system of this molecule is assumed to be that of imidazole.
§ The π electron system of this molecule is assumed to be that of indole.
‖ The π electron system of this molecule is assumed to be that of isoalloxazine. The authors are indebted for this result to Dr. Karreman.

or N_9. Effectively in this molecule, N-methylation at the imidazole ring often results in an N_7 substituted compound (theophylline, caffeine, etc.):



The results for adenine, guanine hypoxanthine, and xanthine indicate that these molecules should be moderately good electron donors but that they should have only very restricted electron-acceptor properties. The best electron donor among these bases should be guanine. This general situation is accentuated in uric acid: the energy of its h.o.m.o. is appreciably higher than that of the other bases (while the energy of its l.e.m.o. remains of the same order of magnitude). *Uric acid should thus be a much better electron donor than the preceding bases.* In fact, it should be a very good electron donor.

A simple way of increasing the electron-donor properties of purines is N-methylation. The degree to which such methylation increases these properties depends on the compound and, for a given compound, on the position of the alkyl group. Thus it can be seen from the figures reproduced in the table that N-methylation should produce only a very slight increase in the electron-donor properties of guanine and that this increase should be practically the same for methylation at N_1 or N_9. In xanthine, methylation at N_1 should also have only a very slight effect on the electron-donor properties of this molecule. On the contrary, methylation at N_3 should increase these donor properties to a very large extent. Intermediate behavior should be observed for methylation at N_7. In uric acid the strongest activation of its electron-donor properties should occur for methylation at N_7, followed by methylation at N_3. Methylation at N_9 should also have a small effect, while that at N_1 should be negligible. The effect of N-methylation on the electron-acceptor properties of purines should be much less important: it should consist, quite generally, in a very slight decrease in these properties.

Although, as previously stated, there is no direct experimental confirmation of the preceding predictions, these considerations and especially those relative to the electron-donor capacity seem to be important for the interpretation of some properties of purines. Such is, in particular, the case of the solubilizing effect of purines on various types of compounds. This effect, which has been studied in respect to aromatic hydrocarbons,[4] aromatic amines,[5] and dibenzacridines,[6] frequently results in the formation of well-defined molecular complexes. The study of the available experimental data indicate, then, that *the solvent power of a series of purines toward, say, a given hydrocarbon runs parallel to the electron-donor properties of the purines.* Thus the solubilizing power (toward 3,4-benzpyrene) of hypoxanthine, xanthine, and adenine is small, while that of guanine is appreciably greater. N-methylation increases the solvent power, and, moreover, the analysis of the experimental results shows that in xanthine the effect of methylation is the strongest when the substitution occurs at N_3 (the next strongest effect being due to a substitution at N_7), while in uric acid it is the methylation at N_7 which has the strongest effect on the solubilizing power. For uric acid the only available data are for di- or polymethyl derivatives, but their analysis shows that the methylation increases the solvent power of the molecule in the order $N_7 > N_3 > N_9 > N_1$. Moreover, a comparison of related polymethylated xanthines and uric acids (e.g., caffeine and 1,3,7-trimethyl-uric acid) indicates that the methylated uric acids have a much greater solubilizing effect than the corresponding xanthines.

These experimental results correlate perfectly with our theoretical calculations on the electron-donor properties of these purines, so that there can be little doubt left as to the importance of their donor properties in determining the solvent power

of the purines.[7] In connection with this result the question may be raised whether the complexes formed between purines and hydrocarbons, amines, or benzacridines are charge-transfer complexes, in the sense defined recently by Mulliken.[8] The problem is being investigated, and the answer to this question will be given, it is hoped, shortly.

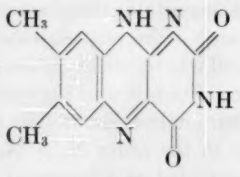
It may be useful to add that the further metabolic degradation products of purines, in animals, should not conserve the strong electron-donor properties of uric acid; e.g., the coefficient K of the energy of the h.o.m.o. in allantoin is equal to 1.

Pyrimidines.—The four fundamental pyrimidines which enter into the constitution of nucleic acids—uracil, thymine, cytosine, and 5-methylcytosine— should be only moderate donors and moderate acceptors. In particular, they should be poorer donors than the fundamental purines.

Furthermore, it may be particularly useful to stress that the highly oxygenated metabolic degradation products of the pyrimidines should behave entirely differently, from the point of view considered here, than the corresponding products of purines. Thus, in striking difference to uric acid, barbituric acid and alloxane have a much lower-lying h.o.m.o. than the original pyrimidines ($K = 1.033$ for both molecules). They should consequently be very bad electron donors.

The coupling of purines and pyrimidines through hydrogen bonding such as the one which occurs in the nucleic acids, following the Watson and Crick model, should have only a very small, if any, influence on the electron-donor properties of the purines: the energy of the h.o.m.o. of the adenine-thymine couple is at 0.425, while that of the guanine-cytosine couple is at 0.308 if two hydrogen bonds are considered and at 0.288 if three such bonds are considered.[9]

Aromatic amino acids.—The four fundamental aromatic amino acids which enter into the constitution of proteins—phenylalanine, tyrosine, histidine, and tryptophan—should also be moderate π electron donors and rather poor electron acceptors. Nevertheless, among these molecules, tryptophan is the one which, by far, should be the best π electron donor. This result is in agreement with the experimental findings of Isenberg and Szent-Györgyi,[1] who showed that, out of these four amino acids, tryptophan is the only one which forms an electron transfer complex with riboflavin. The easy formation of such a complex with serotonin (5-hydroxytryptamine) and other serotonin or tryptamine derivatives and with lysergic acid confirms the hypothesis that the transferred electron comes from the π-electron pool of the indole system.

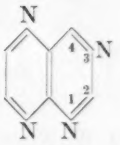


VII

Riboflavin and pteridines.—The striking fact about the isoalloxazine ring (VII), which represents the π electron system of the riboflavin molecule, is that, while in the preceding compounds the energy coefficient of the h.o.m.o. was generally smaller, in absolute value, than the energy coefficient of the l.e.m.o., the reverse is true of

isoalloxazine. Moreover, for this molecule the coefficient of the l.e.m.o. is relatively very small. Consequently, in contrast to all the preceding groups of compounds, riboflavin, while being a moderate π electron donor, should be a good electron acceptor. This is in direct agreement with the experimental results of Isenberg and Szent-Györgyi.[1]

It may also be noted that theoretical computations indicate that an analogous situation should exist to some extent in pteridines. Pteridine itself (VIII) should, in fact, be nearly as good an electron acceptor as riboflavin:



VIII

The same is not true about the 2-amino-4-hydroxypteridine, which represents the basic skeleton of natural pteridines, or about 2,4-diamino- or 2,4-dihydroxypteridines. Nevertheless, even in these molecules the coefficient of the l.e.m.o. is still small enough for these compounds to be relatively good electron acceptors. It may be useful to note in this connection that preliminary experiments by Fujimori seem to confirm the electron-accepting properties of pteridines (in complex formation with tryptophan). Similar properties may then be expected also for folic acid and for pteroic acid.

Addendum.—The existence of only very few experimental data on the ionization potentials of aromatic molecules and the absence of practically any experimental indication as to the value of their electroaffinity makes it very difficult to fix the approximate absolute values of these quantities in the molecules considered here. A very rough evaluation, based on calculations by Matsen for aromatic hydrocarbons,[10] would lead to a prediction of the first π ionization potential of the order of 6–7 ev. for adenine or tryptophan, and of 5 ev. for uric acid and of an electroaffinity of the order of 2 ev. for riboflavin.

This paper was prepared while the authors were visiting scientists at the Institute for Muscle Research at Woods Hole. The authors wish to acknowledge the benefit of many useful discussions on this subject with Drs. Szent-Györgyi, Isenberg, Arnold, and Fujimori. They wish also to thank Dr. Karreman, of the Eastern Pennsylvania Psychiatric Institute in Philadelphia, for permission to use unpublished data on riboflavin.

* This research was sponsored by Grant No. CY 3073 of the National Cancer Institute.

† Permanent address: Laboratoire de Chimie Théorique, Faculté des Sciences, 34 rue d'Ulm, Paris 5e.

[1] I. Isenberg and A. Szent-Györgyi, these PROCEEDINGS, **44**, 857–862, 1958.

[2] A. Pullman and B. Pullman, *Compt. Rend.*, **243**, 380–382, 1956; **246**, 611–614, 1958; **246**, 1613–1616, 1958; *Bull. Soc. Chim.* 766, p. 772, 1958; pp. 973–980, 1958.

[3] For a detailed description of the method see, e.g., B. Pullman and A. Pullman, *Les Théories électroniques de la chimie organique* (Paris: Masson, 1952).

[4] H. Weil-Malherbe, *Biochem. J.*, **40**, 351–363, 1946.

[5] W. J. P. Neish, *Rec. Trav. Chim.*, **67**, 361–373, 1948.

[6] J. Booth and E. Boyland, *Biochem. et Biophys. Acta*, **72**, 75–87, 1953.

[7] Recent work by H. A. Harbury and K. A. Foley (these PROCEEDINGS, **44**, 662–665, 1958) shows that caffeine behaves similarly to tryptophan in giving up an electron when complexing with riboflavin. This gives good evidence of the electron-donor properties of this purine.

[8] R. S. Mulliken, *J. Am. Chem. Soc.*, **72**, 600–608, 1950; 811–820, 1952; *J. Phys. Chem.* **56**, 801–822, 1952; *Rec. Trav. Chim.*, **75**, 845–854, 1956.

[9] Following the proposition by L. Pauling and R. B. Corey, *Arch. Biochem. Biophys.*, **65**, 164–181, 1956.

[10] F. A. Matsen, *J. Chem. Phys.*, **24**, 602–606, 1956; R. M. Hedges and F. A. Matsen, *J. Chem. Phys.*, **28**, 950–953, 1958.

SEROTONIN RECEPTORS. I. EXTRACTION AND ASSAY OF A SUBSTANCE WHICH RENDERS SEROTONIN FAT-SOLUBLE

BY D. W. WOOLLEY*

THE ROCKEFELLER INSTITUTE NEW YORK 27, NEW YORK

Communicated September 26, 1958

INTRODUCTION

Although most biochemists, physiologists, and pharmacologists believe that hormones as well as many drugs act on cells by combining with specific receptors, this "receptor concept" has never enjoyed more than circumstantial support. The existence of receptors has been postulated from indirect evidence, such as the actions of certain drugs which more or less specifically antagonize the effects of a given hormone on living tissues. Also, the receptor hypothesis offers a simpler picture and one more readily understood than certain others which have been put forward to account for the facts. Nevertheless, no one has ever had a hormonal receptor in solution or isolated as a pure chemical compound. This inability to isolate, or even to assay for[1] receptors has led to a growing disbelief in their existence, so that professors of pharmacology are occasionally heard to maintain that receptors are only figments of the imagination. If a way could be found to test for a receptor in vitro without resort to an organized cell or tissue, the general concept would receive much needed support, and it would become possible to isolate in pure form and to define chemically these somewhat nebulous structures.

Recent findings about the mode of action of the hormone serotonin[2] have allowed the development of an assay procedure for a substance which can be extracted from animal tissues and which seems to act in such a way as to suggest that it may be a serotonin receptor, or a piece of it. Serotonin causes muscles to contract. It likewise exerts an effect on the central nervous system,[3, 4] causing, among other things, certain kinds of cells in the brain to contract.[5] Starting with the idea put forward by Csapo[6] to explain the actions of oxytocin and certain other hormones on the uterus, Woolley[2] evolved the idea that serotonin may cause smooth muscles (and certain brain cells) to contract by "opening a special valve for the transport of calcium ions through the lipoidal membrane of the muscle cells." Some experimental evidence in support of such a view was produced. According to these ideas, then, the principal factor which makes the muscle contract would be the flux of Ca^{++} into the actomyosin plus adenosine triphosphate within the

muscle cell. Serotonin and the other hormones which cause contractions would be pictured as doing so because they cause this flow of Ca^{++}. Because the cell membrane is believed to be largely lipoidal and because ionized calcium is not soluble in fats, these ions can pass into the cell only by "leakage" through the non-lipoidal part of the membrane or by some sort of active transport. Woolley conceived of this active transport as occurring in several ways. One of these was for serotonin to combine with a constituent of the cell membrane and the complex so formed to form a fat-soluble calcium salt or chelate. When this complex diffused through the lipid membrane to the interior side, an enzyme was pictured as being present to act upon and cleave some grouping from it, whereupon the complex would disintegrate, liberating Ca^{++}, and serotonin. Other hormones, such as acetylcholine, could be pictured as combining with other specific membranal substances and with Ca^{++} to provide other, but similar, means of getting these ions inside the muscle cell.

If this concept is valid, then it should be possible to extract from muscle cells a lipid with the following unique properties. It should cause serotonin to join with it and become soluble in fat solvents in the presence of calcium ions and at physiological pH. Serotonin itself is not extractable into fat solvents either in the presence or in the absence of calcium ions. Consequently, one should be able to test for such a substance in extracts of muscle merely by shaking the substance in a suitable fat solvent with an aqueous solution of serotonin and Ca^{++} at pH 7–8. Determination of the amount of serotonin which passes into the fat solvent should provide a quantitative, as well as a qualitative, measure of the substance. This is the basis of the work to be described in this paper. Debate about whether this is a piece of the serotonin receptor of smooth muscle will be reserved for the discussion section. For the sake of brevity, however, the substance which can be extracted from certain tissues and which makes serotonin become soluble in fat solvents will be called "receptor substance" in the experimental part of this paper.

EXPERIMENTAL

Extraction Methods.—Two methods for extraction of the receptor substance from tissues have been found successful. (*a*) Immediately after death of an animal the tissue to be examined was excised and cut with sharp scissors into pieces about 1 mm. to a side. As these were cut, they were allowed to fall into a mixture of 2 volumes ether and 1 volume ethanol. For each gram of tissue, 15 ml. of solvent were used. The mixture was then stored at $-16°$ for 24 hours with occasional shaking. It was then filtered, the tissue residue was washed with ether-ethanol (2:1), and the extract was evaporated under reduced pressure in a rotary flash evaporator below 30° to dryness in a 250-ml., round-bottomed flask. The material so obtained was used at once for assay, or if it was to be stored, it was kept at $-16°$. (*b*) In the second method a mixture of chloroform and methanol was used for extraction. The tissue was excised from the animal immediately after death and used at once for extraction or for storage at $-16°$. Frozen tissues were never allowed to thaw because this would allow the receptor substance to come in contact with the enzyme which destroys it. Disruption of the cells by ice crystals would allow this to take place if tissue were allowed to thaw even for a short time. The tissue, either fresh or frozen, was placed in 10 volumes of chloroform-

methanol (2 vols. : 1 vol.) at $-16°$ in a Waring Blendor and ground for 3 minutes. The suspension was then filtered and washed with more of the same solvent. The extract was then concentrated under reduced pressure and handled as in method *a*. Method *b* gave slightly higher yields of the receptor substance from the same tissues but was more expensive for large-scale operations than was *a*.

Irrespective of which method was used, great care was taken to insure that the extracts were perfectly clear. If any particles of tissue were allowed to remain in the extract, these caused destruction of the receptor substance when the solvent was removed and water was added for the assay. The enzyme which attacks the receptor substance probably remained in these particles of extracted tissue.

Assay Method.—An amount of extract sufficient to bind 10–20 μg. of serotonin was used. This came from 5–30 gm. of tissue, depending on the richness of the source. The extracted material was contained in a 250-ml., round-bottomed flask which had contained the extract during the evaporation of the solvent in the extraction step. Benzene (2.5 ml.) and *n*-butanol (2.5 ml.) were added, and, when most of the fat had dissolved, 4 ml. of low-calcium Ringer's solution containing serotonin (5-hydroxytryptamine creatinine sulfate[7] 25 μg/ml. and $CaCl_2$ 5 mg/ml were added. The low-calcium Ringer's solution contained NaCl 9 gm., KCl 420 mg., $NaHCO_3$ 500 mg., glucose 500 mg., $CaCl_2$ 20 mg., and distilled water 1,000 ml., and was made fresh daily. The mixture of benzene-butanol and aqueous solution was shaken thoroughly, poured into a tube, and centrifuged until the top layer was perfectly clear. It was very important that none of the aqueous phase remained in the top layer, giving it a turbidity, because, since the lower phase was rich in serotonin, any trace of it which might remain in the organic layer would give fictitiously high values in the final estimation.

Four milliliters of the organic phase were removed with a pipette and transferred to a clean 10-ml., glass-stoppered centrifuge tube, containing 5 ml. of 0.2 *N* HCl. The tube was then shaken thoroughly and centrifuged until the top layer was perfectly clear. This was discarded, and the aqueous phase was washed with 5 ml. of benzene which was also discarded after centrifugation. The washed acidic aqueous phase now contained the serotonin, which, at physiological pH in the first step, had been rendered soluble in the mixture of butanol and benzene. Shaking of this lipid-soluble complex with aqueous HCl caused it to dissociate, and the serotonin and the calcium chloride were thus carried back into the aqueous phase. The final washing of this aqueous acid phase with benzene was for the purpose of removal of the last traces of fat-soluble tissue constituents.

The HCl extract was washed into a 250-ml., round-bottomed flask with 2 portions of water, and enough 5 per cent aqueous $NaHCO_3$ solution was added to bring the pH to 6–6.3 (pale green to bromthymol blue used as an outside indicator). The solution was concentrated to dryness under reduced pressure at 30°. The residue was dissolved in 9 ml. of water, adjusted to pH 8.0 with $NaHCO_3$ and to 10 ml. This solution was assayed for serotonin by one of two methods. The most useful was a pharmacological method using the isolated uterus of the estrogenized rat.[8, 9] The other method was the colorimetric procedure of Udenfriend *et al.*[10] Because the colorimetric procedure was much less sensitive, it was used only for confirmation of the results obtained with the rat uterus. In our hands the uterus method was as precise as the chemical or fluorospectrophotometric methods

and was more rapid and specific. The colorimetric method, however, provided direct *chemical* evidence to confirm the pharmacological results.

The assay of serotonin by the use of the rat uterus was first described by Erspamer[8] and later by Gaddum and Hameed[9] and by many others. The modification which we have used differed in some details, the chief of which was the use of a low-calcium Ringer's solution[2] at 30°. The low calcium and low temperature were important in order to avoid spontaneous contractions of the organ. The uterus was removed from a normal rat treated the day before with estrogen, as described previously.[2]

Controls.—Controls without added tissue extract were carried through the procedure outlined. This was to test the extractability of serotonin into benzene-butanol without intervention of the receptor substance. In no case was detectable serotonin found in the final extract. The method could detect 0.2–0.4 μg. of the 100 μg. added to the extraction system. This negative control was run repeatedly in order to check on the completeness of separation of the phases in the first extraction with benzene-butanol.

Controls were also done which contained the tissue extract but which lacked the serotonin. This was to test whether serotonin was being carried from the tissue to the final solution, i.e., whether the serotonin actually found in the final solution came from the 100 μg. added at the beginning. In these experiments no detectable serotonin was found. This indicated that serotonin determined in the final solution when an active tissue had been used actually came from the added serotonin and not from the tissue. Such controls were important, because most tissues found to contain receptor substance were known to be rich sources of serotonin.

Units.—A unit of serotonin receptor substance was defined as the amount of material which under the conditions outlined above, would cause 1 μg. of serotonin[11] to appear in the final HCl extract. In other words, a unit was the amount of material which would render approximately 1 μg. of serotonin soluble in a fat solvent such as butanol-benzene (1:1). Only four-fifths of the butanol-benzene phase was used for the recovery of the complexed serotonin, so that, strictly speaking, 1.25 μg. of serotonin were bound by one unit of the receptor substance, but only 1 μg. (i.e., four-fifths) was extracted into the aqueous HCl. The unit thus took cognizance of the aliquot.

Combining Ratios.—The amount of serotonin rendered fat-soluble and re-extractable into aqueous HCl was proportional to the amount of tissue extract used, within certain limits. Thus a chloroform-methanol extract of hog duodenum was divided into two aliquots, one twice the other. When these were carried through the recommended procedure, the smaller aliquot was found to yield 5 μg. of serotonin, and the larger one 10 μg. Similarly, two aliquots of hog colon extract, one twice the other, gave 4 and 1.6 μg. of serotonin, respectively. It was thus clear that in these ranges of concentration the combining ratio of serotonin and tissue constituent was constant. However, with several tissues, aliquots expected to yield only small amounts of serotonin (less than 2 μg.) or large amounts (more than 40 μg.) frequently gave less than the expected amounts. For this reason the range recommended earlier in this paper (10–20 μg. of serotonin bound) was adopted.

The reasons for lack of constant combining ratios at low and high levels of the receptor substance were not entirely clear. At high levels one of the reasons was

the lack of complete extraction of the serotonin-receptor complex. In these cases a second extraction of the aqueous phase with benzene-butanol was found to yield additional serotonin. By restriction of the amount of receptor substance used, multiple extractions were avoided. The low recovery when too small an amount of receptor substance was used was due partly to degradation of serotonin during manipulation of very dilute solutions. A thorough study of combining ratios was postponed until highly purified receptor substance had been isolated. The existing data, however, suggested a stoichiometric reaction between serotonin and receptor substance.

Distribution of the Receptor Substance in Animal Tissues.—Several tissues of various species of animals were assayed according to the methods described above, and the results are summarized in Table 1. Mostly the ether-ethanol extraction

TABLE 1

AMOUNTS OF SEROTONIN RECEPTOR SUBSTANCE EXTRACTED FROM VARIOUS TISSUES BY ETHER-ETHANOL (METHOD *a*) OR CHLOROFORM-METHANOL (METHOD *b*)

Tissue or Material	Method	Receptor Substance (Units per Gm. Wet Weight)
None	*a*	0.0
Rat colon + cecum	*a*	5.0
Rat stomach	*a*	0.8
Rat brain	*a*	1.4
Rat liver	*a*	1.0
Rat leg muscle	*a*	Less than 0.04
Hog colon	*a*	2.0
Hog stomach	*a*	1.3
Hog spinal cord	*a*	1.7
Hog heart	*a*	0.9
Hog erythrocytes	*b*	1.0
Hog kidney	*b*	1.5
Hog duodenum	*b*	5.5
Hog spleen	*b*	1.0
Hog diaphragm	*b*	0.1
Hog lung	*b*	3.3
Clam (*Venus mercenaria*)	*a*	0.2
Mussel (*Mytilus edulis*)	*a*	0.05
Sea scallop	*a*	Less than 0.02
Refined corn oil (Mazola)	..	0.4
Corn seeds	*b*	1.8
Corn leaves (fresh)	*b*	Less than 0.04
Refined olive oil	..	2.2
Egg yolk	*a*	0.3

was used, but for some tissues both methods were compared, and for some only the chloroform-methanol solvent was used. For hog stomach and hog spinal cord, where both methods were used, it was found that ether-ethanol gave 1.3 units/gm, whereas chloroform-methanol yielded 1.6 units/gm for the stomach. For the spinal cord the values were 1.7 (ethanol-ether) and 4.0 (chloroform-methanol).

In general, those tissues which were known to contain much serotonin or to respond readily to it[12] (stomach, intestine, nerve) contained the receptor substance, while those not thus involved with this hormone (voluntary muscle) did not contain detectable amounts. However, the variety of tissues was small, and one would not be surprised to find some exceptions to a generality of this sort if sufficiently large numbers of organs were to be examined. Nevertheless, the correlation, insofar as it went, was noteworthy.

Need for Calcium Ions.—Two equal aliquots of an ether-ethanol extract of rat

colon plus cecum, each obtained from 10 gm. of tissue, were carried through the assay procedure. One was treated in the usual way. The second was done in a medium from which all Ca^{++} had been omitted but which contained all the other constituents. The aliquot containing no Ca^{++} was found to yield 67 μg. of serotonin, while the one worked up in the presence of Ca^{++} yielded 50 μg.

This experiment failed to give evidence that Ca^{++} was needed for the formation of the complex. However, the crude tissue extract contained much Ca, so that mere exclusion of added Ca^{++} was not a sufficient test of whether Ca^{++} entered into the formation of the complex. The final HCl extract contained Ca^{++} as well as serotonin, but, since this Ca^{++} appeared whether or not serotonin had been added to the original mixture, it was not possible to decide whether or not the serotonin-receptor complex required the participation of Ca^{++}. The isolation of purified receptor substance will allow examination of this question.

The working hypothesis does not demand the presence of Ca^{++} for the formation of the serotonin-receptor complex. The formation of this complex is postulated to take place followed by the union with Ca^{++} to give a fat-soluble compound for the transportation of Ca^{++}. Neither serotonin alone nor the receptor substance alone thus renders Ca^{++} fat-soluble and able to be transported in the way pictured. The failure to demonstrate the need for Ca^{++} in the above experiment, therefore, in no way invalidates the working hypothesis because theory does not demand it and calcium was actually present during the experiment.

Choice of Solvent for the Complex.—In order to find a suitable solvent for the extraction of the complex of serotonin and the receptor substance, aliquots of ether-ethanol extract of rat colon plus cecum, each derived from 10 gm. of fresh tissue, were carried through the usual procedure except that benzene-butanol was replaced by other solvents. Controls were also run in which the tissue extract was omitted. This was done in order to determine whether the particular solvent would extract free serotonin. Results are shown in Table 2. It is clear that just any fat solvent

TABLE 2

EXTRACTABILITY OF SEROTONIN INTO VARIOUS FAT SOLVENTS IN PRESENCE OF RECEPTOR SUBSTANCE FROM RAT COLON PLUS CECUM

SOLVENT	SEROTONIN EXTRACTED	
	With Receptor (μg.)	Without Receptor (μg.)
Chloroform	0.8	Less than 0.6
Ethyl acetate	Less than 0.25	Less than 0.25
Benzene	2.7	Less than 0.6
Benzene-butanol (1:1)	50	Less than 0.3

was not sufficient and that benzene-butanol was the best of those tried. A solvent such as pure butanol or amyl alcohol was not suitable because some free serotonin was taken up by these.

Assay of Known Materials.—Many known lipids were assayed by the procedure described, and the results with some are summarized in Table 3. Attention was centered on lipids because the studies on the fractionation of the receptor substance have shown that it was soluble in several dry fat solvents. The commercially available compounds of Table 3 were purchased from reliable sources. More exotic materials, such as monocholesterol phosphate,[13] estradiol diphosphate,[14] soybean lipositol,[15] and beef lung sphingomyelin[16] were synthesized or isolated from

TABLE 3

ASSAY OF VARIOUS LIPIDS FOR SEROTONIN RECEPTOR SUBSTANCE ACTIVITY

Lipid	Receptor Substance* (Units per Gm.)
Cholesterol	Less than 2.7
Monocholesterol phosphate (Na salt)	20
Estradiol	8
Estradiol di-phosphate (Na salt)	Less than 25
3-Benzoylestradiol monophosphate (Na salt)	Less than 30
Hog brain cerebrosides, crude	40
Soybean lipositol	60
Beef lung sphingomyelin, crude	150
Beef brain sphingomyelin (analytically pure)	Less than 40
Beef heart sphingomyelin (analytically pure)	Less than 20

* Values quoted as "less than" mean that no activity was found at the level tested.

natural sources by published procedures. The crude sphingomyelin was a material carried up to the saponification step in Thannhauser's procedure.[16] The cerebrosides were isolated by the barium-acetone method of Loening and Thierfelder.[17] Analytically pure samples of beef heart sphingomyelin and beef brain sphingomyelin were generously supplied by Dr. S. J. Thannhauser.

None of the materials tested showed activity high enough to indicate that the receptor substance could be identified with it. The crude sphingomyelin preparation was the most active (150 units/gm), but, since rat colon plus cecum showed 5 units/gm wet weight or 20 units/gm dry weight (Table 1), this organ would have to contain 13 per cent of sphingomyelin to account for its activity in terms of sphingomyelin. Furthermore, if the receptor substance were to combine with serotonin in equimolecular proportions, one would expect an activity of about 10^6 units/gm if the molecular weight were about 400, or 10^5 if the molecular weight were about 4,000. Finally, highly purified sphingomyelin showed no detectable activity. From considerations such as these it would seem that sphingomyelin is not the receptor substance. The activity of the partially purified material must have been due to impurities. The small activities found for certain other lipids may likewise have been due to impurities, or they may reflect some resemblance of these substances to the receptor substance.

Purification of the Receptor Substance.—The results of the following purification scheme showed that the serotonin receptor was contained in the phospholipid fraction and that its solubility characteristics were such as to make it seem unlikely that phosphatidyl serine or the inositol-containing phosphatides were the active materials. The possibility that the serotonin receptor substance was lecithin was remote because rich sources of lecithin such as egg yolk (see Table 1) were poor sources of the receptor substance.

Fresh hog duodenum (260 gm.) was extracted with choloroform-methanol (2.6 liters) at −16° according to method *b* above. The extract was evaporated under reduced pressure in a flash evaporator to the creamy stage. Acetone (600 ml.) was added, and the resulting suspension was again evaporated to dryness at 30°. Acetone (260 ml.) was added, the precipitate was thoroughly triturated, and the suspension was stored overnight at −16°. The precipitate was collected by centrifugation and washed with cold acetone. This precipitate was dissolved in chloroform (50 ml.). and methanol (520 ml.) was added. The suspension was

stored at −16° overnight, and the precipitate was centrifuged and washed with cold methanol. The methanol was removed from the soluble portion in a flash evaporator at 30°. This methanol-soluble portion represented the active fraction. At each step all fractions were assayed for serotonin receptor activity. The results are summarized in Table 4. The solubility in methanol indicated that the various cephalins, such as phosphatidyl serine and the inositol-containing phosphatides, were probably not the active substances. Speed in operation and the maintenance of low temperatures were essential during the operations because the receptor substance proved to be rather unstable. Thus, when an acetone precipitate fraction was stored in chloroform at −16° and assayed at weekly intervals, it was found that the activity fell to one-fourth of the original after 4 weeks. In aqueous suspensions the decomposition was much more rapid.

TABLE 4

PARTITION OF SEROTONIN RECEPTOR SUBSTANCE ACTIVITY DURING FRACTIONATION OF HOG DUODENAL EXTRACT

SOLVENT	TOTAL ACTIVITY IN: Precipitate (Units)	Filtrate (Units)
Chloroform-methanol extract	...	1,430
Acetone	1,040	110
Methanol	150	1,350

This same fractionation scheme and various modifications of it, using benzene, ethanol, and petroleum ether after the acetone precipitation step, have been tried on four batches of hog duodenum, on three batches of hog stomach, one batch of hog spinal cord, and one batch of hog colon. All these experiments have shown that the receptor substance is precipitated by acetone and that the recovery of units of activity in the various fractions was satisfactory when cold dry solvents were used.

DISCUSSION

The data of this paper show that a substance can be extracted from certain tissues which will render serotonin soluble in benzene-butanol in the presence of Ca^{++}. The substance itself is soluble in various fat solvents and must therefore be regarded as a lipid. The existence of such a substance would be anticipated if the earlier conclusions of Woolley[2] are followed for the mechanism of action of serotonin on smooth muscles. This and the demonstration of the occurrence of the substance in those tissues which respond to serotonin and its absence from some which do not would tend to suggest that it may be a part of the serotonin receptor of cells. However, no satisfying proof of such an idea is yet available. It is merely a plausible working hypothesis which accounts for the facts thus far known.

If, however, we grant that the substance studied in this work is a portion of the serotonin receptor, two possibilities are opened. One is the isolation of this piece of the receptor in chemically pure condition and establishment of its structure, a thing which has not been possible with any hormonal or drug receptor previously. The other is the possibility of understanding in terms of precise chemical reactions the mode of action of this hormone. It would therefore seem advisable to investigate this substance further.

SUMMARY

A substance was extracted from stomach, colon, brain, spinal cord, and certain other tissues by the action of cold fat solvents. This substance had the property of making serotonin become soluble in a fat solvent (benzene-butanol) in the presence of Ca^{++}. A method of assay for this substance was described, and its distribution in tissues was studied. The assay depended on the extraction of serotonin into benzene-butanol from aqueous solution in the presence of the substance, the decomposition of the complex so extracted, and the measurement of the amount of serotonin thus liberated. Some reasons were given for entertaining the idea that this substance might be the nonenzymic portion of the serotonin receptors of smooth muscle and nerve.

* With the technical assistance of N. K. Campbell.

[1] Although indirect assays for receptors can be made with the use of whole animals or organized tissues, what is meant here is that there is no in vitro assay which is independent of a response in an organized cell or mass of cells.

[2] D. W. Woolley, these PROCEEDINGS, **44**, 197, 1958.

[3] D. W. Woolley and E. Shaw, these PROCEEDINGS, **40**, 228, 1954.

[4] D. W. Woolley and E. N. Shaw, *Ann. N.Y. Acad. Sci.*, **66**, 649, 1957.

[5] H. H. Benitez, M. R. Murray, and D. W. Woolley, *Proc. 2d Intern. Cong. Neuropath, London (1955)* (Amsterdam: 1958), Excerpta Medica Foundation, p. 423.

[6] A. Csapo, *Proc. 1955 Laurentian Hormone Conference*, **12**, 405, 1956.

[7] Generously supplied by Dr. K. Hamlin, of Abbot Research Laboratories.

[8] V. Erspamer, *Arch. intern. pharmacodynamie*, **103**, 293, 1953.

[9] J. H. Gaddum and K. A. Hameed, *Brit. J. Pharmacol.*, **9**, 240, 1954.

[10] S. Udenfriend, H. Weissbach, and C. T. Clark, *J. Biol. Chem.*, **215**, 337, 1955.

[11] All weights of serotonin refer to 5-hydroxytryptamine creatinine sulfate and not to 5-hydroxytryptamine free base.

[12] Among the plants, substitute "indoleacetic acid" for "serotonin," as discussed by D. W. Woolley, *Nature*, **180**, 630, 1957.

[13] H. von Euler, A. Wolf, and H. Hellstrom, *Ber. chem. Ges.*, **62B**, 2451, 1929.

[14] *Soc. pour l'ind. chim. à Bâle, C.A.*, **41**, 2450, 1947.

[15] D. W. Woolley, *J. Biol. Chem.*, **147**, 581, 1942.

[16] S. J. Thannhauser, J. Benotti, and N. F. Boncoddo, *J. Biol. Chem.*, **166**, 669, 1946.

[17] H. Loening and H. Thierfelder, *Z. physiol. Chem.*, **74**, 282, 1911.

---

*SUCROSE AND GROWTH OF EXCISED ROOTS OF AN INBRED ZEA MAYS**

BY WILLIAM J. ROBBINS

NEW YORK BOTANICAL GARDEN AND DEPARTMENT OF BOTANY, COLUMBIA UNIVERSITY

Communicated October 20, 1958

In experiments on the cause of hybrid vigor, excised root tips of two inbred strains of *Zea mays* and their F_1 hybrid[1] were cultivated on a mineral salt–sucrose medium[2] solidified with 1.5 per cent agar. The corn grains were sterilized with Chlorox and germinated on water agar at 25° C. When the roots had reached a length of 2 or 3 cm., the apical portion of the root, 3 or 4 mm. in length, was excised,

transferred to a Petri dish containing 20 ml. of the agar medium, and incubated in the dark at 25°.

The root tips of one of the inbred strains, Oh 43, grew but little on this medium; in fact, with an occasional exception, growth ceased within a day or two and the final length was no more than two or three times the original. The root tips of the other inbred and the F_1 hybrid increased in the course of 11 or 12 days some eight to twelve times the original length, and some produced branch roots.

The addition of yeast extract, gibberellic acid,[3] a mixture of thiamin, pyridoxine, and nicotinic acid or a mixture of the known B vitamins plus purine and pyrimidine bases to the basal mineral salt–sucrose agar medium had little or no beneficial effect on the root tips of Oh 43.

When a root tip of Hy 2 was cultivated alongside that of Oh 43, a substantial amount of growth was induced in the latter (Fig. 1). A root tip of the F_1 hybrid

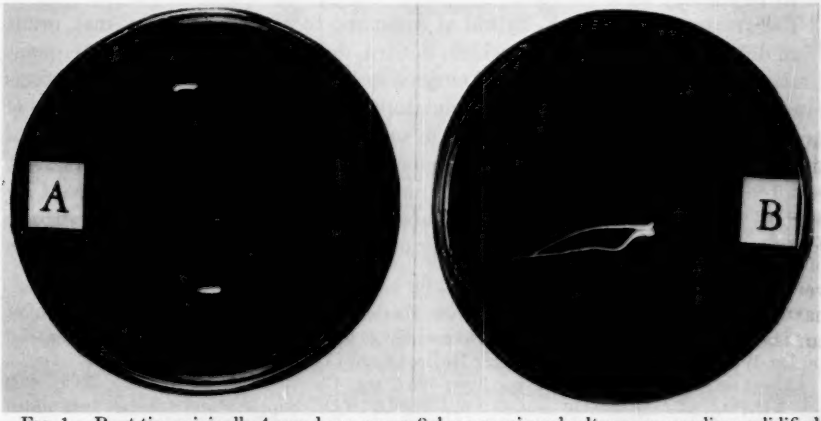


FIG. 1.—Root tips originally 4 mm. long, grown 9 days on mineral salt–sucrose medium solidified with 1.5 per cent agar. *A*, root tips of inbred *Zea mays*, Oh 43. Final length 6 mm. *B*, Oh 43 above, Hy 2 below. Final lengths 27 and 39 mm., respectively. Note effect of presence of Hy 2 root tip on growth of root tip of Oh 43.

had the same effect, but two root tips of Oh 43 cultivated side by side had no effect on one another.[4] We assumed that the root tips of Hy 2 and of the F_1 hybrid excreted an unidentified and potent growth substance into the agar and that this substance was required for the growth of the Oh 43 root tips which were unable to synthesize it.

The assumption was supported by the favorable effect of the extracts of germinated grains on root tips of Oh 43. Water extracts of Hy 2 and the F_1 hybrid added to the basal medium increased the growth of root tips of Oh 43. Extracts of Oh 43 grains also had a favorable effect, though less so, on an equivalent grain basis, than those of Hy 2 or the F_1 hybrid. The beneficial action of the extract of grains of Oh 43 was expected because grains of Oh 43 germinated and the primary root grew; this could not happen unless the hypothetical material came from the grain. It seemed reasonable to suppose that the unidentified growth substance was synthesized by the shoots of Oh 43 and stored in the grains, but to a smaller degree than for the other two strains.

However, the substitution of dextrose for sucrose in the mineral salt–sugar medium also induced growth in the root tips of Oh 43. By tests with Fehling's solution we found sucrose to be inverted in the vicinity of the root tips of Hy 2 and of the F_1 hybrid. We concluded, therefore, that the root tips of Oh 43 were unable to use sucrose and that the extracts of germinated grains were favorable because they contained dextrose derived from the hydrolysis of starch. The presence of a root tip of Hy 2 or of the F_1 hybrid favored the growth of root tips of Oh 43 on a sucrose medium because the former inverted the sucrose by the excretion of invertase, thus making the carbohydrate available.[5]

The inability of the excised root tips of Oh 43 to invert sucrose suggests that, as far as carbohydrate metabolism is concerned, sucrose plays no role in plants of this strain and that sucrose as such would not be found in their tissues. This appears probable, though it does not necessarily follow, since the top of the plant may differ in its physiology from the root.

The greater vigor of an F_1 hybrid as compared to its inbred parent, may result from differences in metabolism, which, in turn, depend upon qualitative or quantitative differences in the activity of enzyme systems. What those enzyme systems are, whether they produce growth stimulators, are responsible for the elimination of growth inhibitors, or influence growth in some other way bears on the problem of growth in general as well as on the specific problems involved in the development of inbreds and their F_1 hybrids.[6] It is unlikely, however, that a deficiency of invertase in one inbred parent and the inability of that parent to assimilate sucrose has any direct significance as far as the causes of hybrid vigor in the F_1 hybrid are concerned.

* The assistance of Mary Christine Howson is acknowledged with appreciation.

[1] The grains of the inbred strains (Oh 43 and Hy 2) and their F_1 hybrid were kindly supplied by Dr. William L. Brown, of the Pioneer Hi-Bred Corn Company.

[2] The basal medium contained, per liter, 166.5 mg. $Ca(NO_3)_2 \cdot 4H_2O$; 41.6 mg. KCl; 83.2 mg. KH_2PO_4; 83.2 mg. KNO_3; and 83.2 mg. $MgSO_4 \cdot 7H_2O$. Supplemental minerals were added in p.p.m. as follows: 0.5 Fe as Fe tartrate; 0.05 B; 0.05 Mn; 0.005 Zn; 0.002 Cu; and 0.005 Mo.

[3] Gibberellic acid at 1 μg/ml stimulated the production of branch roots by the excised roots of Hy_2 and the F_1 hybrid grown on the basal agar medium. It had a similar effect on excised roots of a flint corn and Vaughn's Golden Cross Bantam sweet corn.

[4] Root tips of Oh 43, Hy 2, and the F_1 had no effect, as determined by our methods, on those of Hy 2 or those of the F_1.

[5] Many of the Mucoraceae, especially of the genus *Rhizopus*, form no invertase and cannot utilize sucrose. They grow vigorously on invert sugar.

[6] See discussion by W. Gordon Whaley, by William J. Robbins and others in *Heterosis*, ed. F. L. Gowan (Ames, Iowa: Iowa State College Press, 1952).

THE VISCOSITY OF HIGH POLYMERS—THE RANDOM WALK OF A GROUP OF CONNECTED SEGMENTS

By Henry Eyring, Taikyue Ree, and Nishio Hirai

Department of Chemistry, University of Utah, Salt Lake City, Utah

Communicated October 20, 1958

In this paper, the relaxation theory of viscosity is generalized to include the complications arizing with large molecules, which tangle in concentrated solutions and in a pure polymeric system.

I. *The Viscosity of a Simple Substance.*—We first review briefly elementary relaxation theory. By definition, the viscosity is the ratio of shear stress, f, to rate of shear, $\dot{s}$. By reference to Figure 1 we write:

$$\eta = \frac{f}{\dot{s}} = f/\left\{\frac{\lambda}{\lambda_1} 2k' \left(e^{f\lambda\lambda_2\lambda_3/2kT} - e^{-f\lambda\lambda_2\lambda_3/2kT}\right)\right\}$$

$$= f/(\lambda/\lambda_1)2k' \sinh (f\lambda\lambda_2\lambda_3/2kT). \tag{1}$$

Here f is multiplied by the area, $\lambda_2\lambda_3$, of the flow unit in the shear plane to get the force acting on a flow unit, and this acts through a distance, $\lambda/2$, to lower the activation energy by $f\lambda\lambda_2\lambda_3/2$ in the direction of shear, while the activation energy is raised by this same amount for motion against the shear force. Thus the number of jumps in the shear direction is k' exp $(f\lambda\lambda_2\lambda_3/2kT)$, and in the backward direction it is k' exp $(-f\lambda\lambda_2\lambda_3/2kT)$, giving a net number of jumps, k' $\{$ exp $(f\lambda\lambda_2\lambda_3/2kT)$ − exp $(-f\lambda\lambda_2\lambda_3/2kT)\}$, in which λ_1 is the distance between centers of gravity of the relaxing units lying on the two sides of the shear plane. Equation (1) gives a very satisfactory account of the viscosity of simple liquids.[1]

II. *The Viscosity of Solutions.*—When there is more than one type of relaxing system, the ith type may be assumed to occupy a fraction, x_i, of the shear surface and is acted on by a shear force, f_i, per unit area. In such a case, one finds for the viscosity the relationship[2]

$$\eta = (\sum_i x_i f_i)/\dot{s} = \sum_i \frac{x_i\beta_i}{\alpha_i} \frac{\sinh^{-1} \beta_i\dot{s}}{\beta_i\dot{s}} \tag{2}$$

The result

$$\dot{s} = \frac{\lambda}{\lambda_1} 2k' \sinh\frac{f\lambda\lambda_2\lambda_3}{2kT} = \frac{1}{\beta} \sinh \alpha f \tag{3}$$

was used in equation (1). When equation (3) is solved for f, one obtains $f = (1/\alpha)$ $\sinh^{-1} \beta\dot{s}$. In a complex system, this takes the form

$$f_i = \frac{1}{\alpha_i} \sinh^{-1} \beta_i\dot{s} \tag{4}$$

for the ith unit. This result for f_i leads to the final equality (2).

III. *The Viscosity of a Pure High Polymer.*—Consider next the application of equation (1) to a pure high polymer system having a molecular weight, M, with kinetic segments of mass, m_1, so that there are $n_1 = M/m_1$ kinetic segments in a

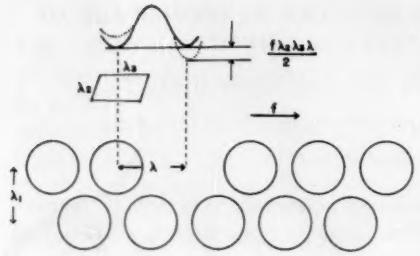


FIG. 1.—Flow in a simple liquid

molecule which jump independently except for the restriction that they remain tied together. The section *ab* in Figure 2 represents such a kinetic segment. Further we designate by m_2 the mean mass of the tangle segment, which is the part of a molecule lying between any two neighboring entangling molecules, *A* and *B*, etc. (cf. Fig. 2). Such a long molecule is obliged to circle (slalom) around $n_2 - 1$ cross-lying molecules in order that its center of gravity can advance or retreat along the shear plane. The molecule is then said to be divided up into $n_2 = M/m_2$ tangle segments, each of which, at any instant, must pursue a different objective, i. e., pass around its particular tangle point.

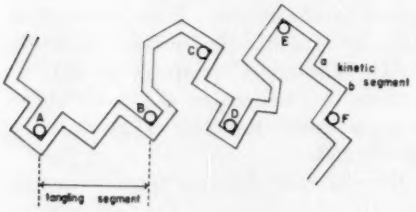


FIG. 2.—Flow in a high polymeric system. The polymer molecule flows by a slalom motion through the tangling molecules, *A*, *B*, *C*, *D*, etc.

Let η_1 designate the viscosity of a system made up of unlinked kinetic segments, while η_p designates the viscosity of a high polymer in which n_1 kinetic segments are linked together.

Now, first, η_p has an extra factor $(n_1)^{1/2}$ in it due to the fact that the distance between centers of gravity, λ_1, increases with the volume of the high polymer molecule (and therefore with the molecular weight) to the one-third power. Second, since the center of gravity is displaced each time any segment jumps, the rate of displacement for the center of gravity is n_1 times the rate constant, k', for a segment. Third, whereas the jump of a segment displaces the segment by a length, λ, it displaces the center of gravity of the polymer molecule, on the average, by only the distance, λ/n_1n_2. The factor $1/n_1$ arises from the fact that a displacement of a single segment displaces the center of gravity of n_1 segments by only $1/n_1$th as much as the single segment is displaced. The factor $1/n_2$ comes from the fact that only the kinetic segments lying between one pair of entangling molecules can independently act to advance the center of gravity in the shear direction. The other tangling segments are then obliged to find their way around randomly located tangling points, so that their displacements cancel out, on the average, with respect to any effect they might have in advancing the center of gravity along the shear sur-

face. The surface area of the kinetic segment, $\lambda_2\lambda_3$, is not changed by joining the segments together.

Introducing these three factors into equation (1) gives

$$\eta_p = \frac{f n_1^{1/3} \lambda_1}{(\lambda/n_1 n_2) 2k' n_1 \sinh (f\lambda\lambda_2\lambda_3/n_1 n_2 2kT)}. \tag{5}$$

For not too large stresses, the hyperbolic sine can be replaced by its argument, so that, for this Newtonian case, equation (5) becomes

$$\eta_p = \frac{kT}{\lambda_2\lambda_3\lambda^2 k'/\lambda_1} n_1^{4/3} n_2^2 = \eta_1 \frac{M^{10/3}}{m_1^{4/3} m_2^2}. \tag{6}$$

When the polymer molecule is not so large, i. e., $M \leq m_2$, the afore-mentioned tangle effect does not appear. In this case, equations (5) and (6) become, respectively,

$$\eta_p = \frac{f n_1^{1/3} \lambda_1}{(\lambda/n_1) 2k' n_1 \sinh (f\lambda\lambda_2\lambda_3/n_1 2kT)} \tag{7}$$

and

$$\eta_p = \frac{kT}{\lambda_2\lambda_3\lambda^2 k'/\lambda_1} n_1^{4/3} = \eta_1 \frac{M^{4/3}}{m_1^{4/3}}. \tag{8}$$

According to the above results, the viscosity of a high polymeric system is proportional to $M^{10/3}$ if the molecular weight, M, is larger than a critical value, m_2, whereas the viscosity is proportional to $M^{4/3}$ for unentangled molecules. By reviewing many experimental results, Fox, Gratch, and Loshaek[3] showed that in the high-molecular-weight region viscosity is proportional to $M^{3.4}$, while in the low-molecular-weight region viscosity is proportional to M^x, where x varies between 0.84 and 1.76. Bueche[4a] explained the dependence of viscosity on molecular weight theoretically. According to his theory, the viscosities in the two regions are proportional to $M^{3.5}$ and M, respectively.

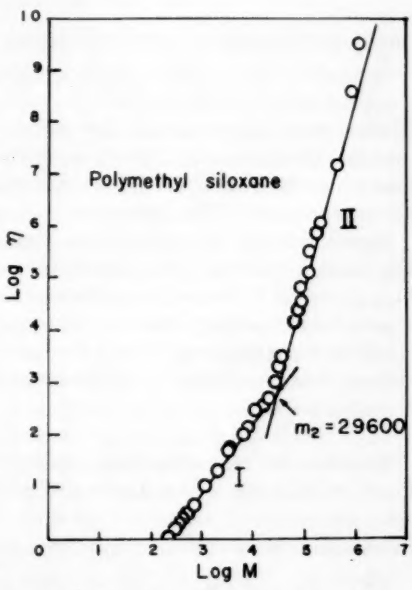


FIG. 3.—The curve of log η versus log M for polymethyl siloxane

IV. *Test of the Proposed Theory.*—As an example, the curve of log η vs. log M for polydimethyl siloxane is shown in Figure 3. Here two straight lines cross at a point at which the molecular weight is m_2. The straight lines, *II* and *I*, are represented by the logarithms of equations (6) and (8), respectively. The test of our theory is made by

TABLE 1

EFFECT OF MOLECULAR WEIGHT ON VISCOSITY OF HIGH POLYMERS

	s_1	s_2	$-A$	$-B$	log m_2 calc.	log m_2 obs.	Temperature (° C)	Reference
Polyisobutylene	1.75	3.40	13.7	6.30	3.70	4.29	217	3, 6
Polystyrene	1.58	3.44	13.6	5.03	4.29	4.68	217	3, 6*a*, 7
Polydimethyl siloxane	1.34	3.70	15.7	5.14	5.28	4.71	25	3, 8, 9, 10, 11
Polymethyl methacrylate*	1.4	3.4	14.2	5.50	4.35	4.20	60	3, 4*b*
Polydecamethylene adipate	1.34	3.40	12.1	4.47	3.82	3.68	109	3, 12
Polydecamethylene sepacate	1.23	3.23	11.4	4.17	3.62	3.67	109	3, 12
Polydiethylene adipate	1.26	2.92	10.0	4.11	2.95	3.56	109	3, 12
Poly (ϵ-caprolactam)								
Linear chain	1.66	3.52	12.8	5.79	3.52	3.78	253	3, 13
Dichain	1.70	3.50	12.5	5.80	3.35	3.72	253	3, 13
Tetrachain	1.31	3.24	11.8	4.29	3.76	3.79	253	3, 13
Octachain	1.31	3.24	12.7	4.75	3.93	4.09	253	3, 13

* 25 per cent solution in diethyl phthalate.

comparing the slopes s_2 and s_1 of the two straight lines in the regions $M \geq m_2$ and $M \leq m_2$ with the theoretically expected slopes, 10/3 and 4/3. In Table 1, s_1 and s_2 in various cases are listed. One may see that the theoretical predictions are about right. Another method of testing is as follows: The straight lines, *II* and *I*, are mathematically represented by the following two equations, respectively,

$$\log \eta = \mathrm{A} + \mathrm{x} \log \mathrm{M}, \tag{9}$$

$$\log \eta = \mathrm{B} + \mathrm{y} \log \mathrm{M}. \tag{10}$$

At the crossing point of equations (9) and (10), one obtains the relation

$$\mathrm{A} = \mathrm{B} - (\mathrm{x} - \mathrm{y}) \log \mathrm{m}_2. \tag{11}$$

If we assume in, accordance with our theory $x - y = 2$, the critical values, m_2, is calculated from equation (11) by using the intercepts, A and B, of the two straight lines on the log η axis. In Table 1, the predicted values of m_2 and the observed values are compared. The agreement is satisfactory.

Experimentally, the value of the slope s_2 is better established than the slope s_1. The average value of s_2, 3.4, which has been generally accepted in the rheology of high polymers,[3, 5] is very close to our theoretical value, 10/3. No generally accepted value has been determined for the average value of s_1, because of the nonconcordant experimental data. Our analyses of the experimental data, however, indicate that the average experimental value of s_1 is 1.4, in agreement with the theoretical value, 4/3.

This research was supported in part by a grant from the Petroleum Research Fund administered by the American Chemical Society. Grateful acknowledgment is hereby made to the donors of the fund. We also wish to express our gratitude to the National Science Foundation for partial support of this work.

[1] H. Eyring, *J. Chem. Phys.*, **4**, 283, 1936; S. Glasstone, K. J. Leidler, and H. Eyring, *The Theory of Rate Processes* (New York: McGraw-Hill Book Co., 1941), pp. 477–551.

[2] T. Ree and H. Eyring, *J. Appl. Phys.*, **26**, 793, 800, 1955.

[3] T. G. Fox, S. Gratch, and S. Loschaek, *Rheology*, ed. F. R. Eirich (New York: Academic Press, 1956), **1**, 431–523.

[4] F. Bueche, (*a*) *J. Chem. Phys.*, **25**, 599, 1956; (*b*) *J. Appl. Phys.*, **26**, 738, 1955.

[5] H. Leaderman, *Annual Review of Physical Chemistry* (Palo Alto, Calif.: Annual Reviews, Inc., 1958), **9**, 179.

[6] T. G. Fox and P. J. Flory, (*a*) *J. Am. Chem. Soc.*, **70**, 2384, 1948; (*b*) *J. Phys. Chem.*, **55**, 221, 1951.

[7] T. G. Fox and P. J. Flory, *J. Appl. Phys.*, **21**, 581, 1950; *J. Polymer Sci.*, **14**, 315, 1954.

[8] A. J. Barry, *J. Appl. Phys.*, **17**, 1020, 1946.

[9] M. J. Hunter, E. L. Warrik, J. F. Hyde, and C. C. Currie, *J. Am. Chem. Soc.*, **68**, 2284, 1946; E. L. Warrick, W. A. Piccoli, and F. O. Stark, *J. Am. Chem. Soc.*, **77**, 5017, 1955.

[10] A. Charlesby, *Proc. Roy. Soc. London*, **A230**, 120, 1955.

[11] R. L. Merker, *J. Polymer Soc.*, **22**, 353, 1956.

[12] P. J. Flory, *J. Am. Chem. Soc.*, **62**, 1057, 1940.

[13] J. R. Schaefgen and P. J. Flory, *J. Am. Chem. Soc.*, **70**, 2709, 1948.

---

*BIOLOGICAL DAMAGE IN DEVELOPING GERM CELLS OF DROSOPHILA VIRILIS IN OXYGEN AND NITROGEN WITH 14-MEV NEUTRONS**

BY MARY L. ALEXANDER*

DEPARTMENT OF BIOLOGY, UNIVERSITY OF TEXAS, M. D. ANDERSON HOSPITAL AND TUMOR INSTITUTE, HOUSTON, TEXAS, AND BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY, OAK RIDGE, TENNESSEE

Communicated by J. T. Patterson, October 2, 1958

Introduction.—Biological damage after irradiation is the result of a complex series of interactions, depending on the quality of the radiation and the characteristics of the biolcgical system. The predominant factors responsible for the damage may be determined by the type of radiation as well as by the physicochemical systems of the biological material. The separation of the factors involved may be accomplished, in part, by using different types of radiation and cells with various biological characteristics.

Different amounts of biological damage, scored as translocations and dominant lethals, have been recovered from the germ cells of *Drosophila virilis* with a series of different radiations, including 200-kvp X-rays, Co^{60} γ-rays, 22-Mev X-rays, and fission neutrons.[1-3] In postmeiotic cells, young spermatids were consistently more sensitive to radiation than were the more mature, non-motile sperm. The resulting variation in radiobiological damage may involve both differences in sensitivity of chromosome breakage and changes in the chemical system of cells. Radiobiological actions obtained with fission neutrons indicate a variation in chromosome breakage,[2] whereas results with X-radiation indicate an involvement of the chemical systems, since environmental changes were more effective for determining the amount of biological damage.[1]

We treated germ cells in spermatogenesis with 14-Mev neutrons in O_2 and N_2 atmospheres. The average linear energy transfer (LET) for 14-Mev accelerator neutrons is about 12–14 Kev/μ, which is equivalent to 400 ion pairs/μ. Biological damage from the densely ionizing radiations of neutrons in oxygen and nitrogen can be compared in sperm, spermatids, and meiotic and spermatogonical germ cell.

Materials and Methods.—The Texmelucan strain of *D. virilis*, 1801.1, was used as a standard stock for testing radiation effects from accelerator neutrons on the de-

veloping germ cells of spermatogenesis. *Drosophila virilis* offers a system in which maturation of germ cells requires a longer period of time than in *D. melanogaster*. This slower rate of development allows more complete separation of the various types of germ cells for testing biological damage. Clayton[4] has described the absolute and relative frequency of germ cells in the testes of larval, pupal, and young imago stages of *D. virilis*. Histological data and biological tests can be compared for a more complete analysis of radiation damage.

Samples of adult males were collected within 2 hours after eclosion and treated at 19–21 hours after eclosion. Sperm bundles were the most mature type of germ cell present in the testes. Coiled sperm do not appear in the testes until 3 days later (4 days after eclosion). The appearance of dense cystlike bodies in the region of spermatocyte and spermatid development approximates the time of appearance of coiled sperm in the lower portion of the testes. The germ cells present at the time of irradiation were sperm bundles, spermatids in various stages of spermiogenesis, spermatocytes, and spermatogonia.

For pupal tests, samples were collected at the prepupal stage before the cuticle became darkly pigmented. Pupae up to 3 hours old were collected. Radiation treatments were started 110–115 hours after pupation. The sample of germ cells included spermatogonia, spermatocytes, spermatids, and small numbers of sperm bundles. The development of larvae and pupae was under optimum conditions.

Sperm samples were obtained by mating treated males, individually, to 3 females. In the adult tests, 2 of the 3 females were heterozygous normal obtained by crosses between two other *D. virilis* stocks, (1999 ♀ × 2375.8 ♂), and 1 female carried homozygous recessive markers *b* on chromosome 2; *tb* and *gp* on chromosome 3; *cd* on chromosome 4; and *pe* on chromosome 5. After each 2-day mating period, the marker females were placed on fresh food. The F_1 males, heterozygous for the treated chromosomes and marker genes, were backcrossed, individually, to marker females. Recombination classes of marker genes were used to determine frequency of translocations between the second, third, fourth, fifth, and Y chromosomes. The total sample for translocations consisted of samples of 14 F_1 males from each P_1 cross.

Dominant lethal tests were obtained from the heterozygous normal females. After the 2-day mating period, the normal females were placed on fresh food. Egg counts were made for individual females each day for a 4- or 5-day period. The percentage of development was calculated from the number of pupae that developed from the total egg sample. Dominant lethals were calculated from the percentage of egg development. The imago from the pupae tests were mated either 1 male to 3 normal females or 1 male to 3 marker females.

Control tests for egg development have been carried out, using 7-day-old-males. Remating periods from young males and imago from pupae were collected in the same way as samples for the radiation tests. Control tests of crosses between males of 1801.1 and heterozygous females (1999 ♀ × 2375.8 ♂) have been sampled for a number of tests and repeated a number of times over the last 2 years.

In tests I-1 and I-2, we used 2 nylon chambers, each divided in half, with O_2 flowing into one compartment and N_2 into the other. Adult males, 19–21 hours old, were divided into 4 groups, and a group was placed in each compartment. A constant flow of gas passed through the chamber for a 30-minute period before,

during, and after neutron treatment. The oxygen test I-1 and the nitrogen test I-2 were on the sample of males treated at the same time. A dose of 999.5 rads of 14-Mev neutrons was given for 30 minutes and 20 seconds at 19–21° C.

For the pupae tests, N_2 and O_2 treatments were started 8 hours before neutron treatment. Oxygen test III-1 and N_2 test III-2 were on the same pupal sample and received 300 rads of 14-Mev neutrons. The total dose was given in 11 hours and 51 minutes at a rate of 25 rads per hour. In test IV, half the pupal sample was treated in O_2 and half in N_2. Both samples were treated at the same time with 196 rads of 3-Mev neutrons. The total dose was given at a constant rate over a 10-hour period.

The 14- and 3-Mev monoenergetic neutrons were produced by the $T(d,n)He^4$ and $D(d,n)He^3$ nuclear reactions, using a 250-Kev Cockcroft-Walton accelerator and thick zirconium hydride targets. If it is assumed that the value for each micron of track area is of equal importance, the average LET value for 14-Mev neutrons is about 12-14 Kev/μ, which is equivalent to about 400 ion pairs/μ. For 3-Mev neutrons, values are about 30 kev/μ and 900 ion pairs/μ. Randolph[5] describes the dosimetry and the physical characteristics of the neutron radiation obtained with the Cockcroft-Walton accelerator at the Biology Division of the Oak Ridge National Laboratory.

Results.—Limits for the samples of germ cells obtained in the various mating periods can be approximated. Period A contained postmeiotic cells treated as sperm bundles. The irregularity of the amount of biological damage in period B indicated that this sample often contained mixtures of sperm bundles and younger spermatids. Periods C, D, and E contained samples of cells treated as spermatids in various stages of spermiogenesis. A peak in the biological damage is usually obtained in mating period E, although maximum increases may often be attained in period D and extend through period E. Definite peaks in lethal damage can be obscured by high doses. The low translocation rate in period F indicates a mixture of meiotic and postmeiotic cells. Dominant lethals are not a reliable measure for separating meiotic and postmeiotic cells, since decreases in the lethals in period F can be eliminated by high doses of X-rays and by lower doses of other types of radiation.[2, 3] Irradiation also produces cell degeneration among meiotic and spermatogonial cells and results in reduction of mature sperm obtained during certain mating periods.[1, 2] Samples from period G indicated that germ cells obtained in this period were the most sensitive to cell degeneration and would represent late spermatogonial cells by comparison with cell degeneration tests for spermatogonial cells of the mouse.[6] Lethal damage drops to a lower level in period H but probably contains mixtures of several spermatogonial types, since an additional 2-day mating period is usually necessary before the lethal values equal those of the control levels.[1, 2] Designation of cell stages has been discussed by Alexander and Stone[1] and correlated with the histological data by Clayton.[4]

Table 1 and Figures 1 and 2 show translocation and dominant lethal damage produced by 14-Mev neutron treatment of young males in O_2 and N_2 atmospheres. The translocations are reported in relation to the total number of sperm containing translocations and are classified into those involving two, three, four, or five chromosomes. In Table 2, translocations are classified into the percentage of these interchanges and indicate the minimum number of breaks.

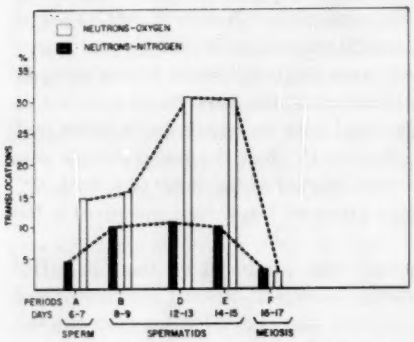


FIG. 1.—Differential effect of 14-Mev neutrons with O_2 and N_2 in different postmeiotic and meiotic germ cell stages.

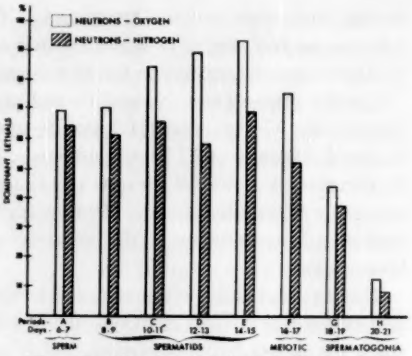


FIG. 2.—Dominant lethal damage recovered from the germ cells of spermatogenesis in O_2 and N_2 after treatment with 14-Mev neutrons. Approximate limits of germ cell samples are indicated.

TABLE 1

TRANSLOCATION AND DOMINANT LETHAL DAMAGE IN OXYGEN AND NITROGEN WITH 14-MEV NEUTRONS

EXPERIMENT	PERIOD*	TRANSLOCATION TYPES T_2	T_3	T_4	T_{2+2}	T_5	TOTAL SPERM TESTED	SPERM WITH TRANSLOCATIONS Per Cent	One Standard Deviation	DOMINANT LETHALS Eggs	Pupae	Per Cent Lethals
	A	59	8	0	0	0	465	14.0	1.6	2,768	865	68.7
	B	75	5	0	1	0	508	15.9	1.7	3,148	945	70.0
	C	..	..	..	..	..	..	..	..	3,025	504	83.3
I-1 Oxygen	D	71	13	4	1	1	285	31.6	2.3	3,836	433	88.7
(999.5	E	35	7	4	0	1	152	30.9	3.9	4,191	317	92.4
rads)	F	3	2	0	0	0	189	2.6	1.3	551	143	74.0
	G	..	..	..	..	..	..	..	..	1,888	1,804	42.6
	H	..	..	..	..	..	..	..	..	382	337	11.8
	A	15	2	0	0	0	433	3.9	0.8	1,917	695	63.8
	B	45	1	0	0	0	457	10.0	1.4	4,207	1,659	60.6
	C	..	..	..	..	..	..	..	..	3,042	1,063	65.1
I-2 Nitrogen	D	22	2	0	1	0	218	11.5	2.3	2,486	1,060	57.4
(999.5	E	28	1	1	0	0	302	9.9	1.7	2,750	891	67.6
rads)	F	9	0	0	0	0	302	3.0	1.0	656	324	50.6
	G	..	..	..	..	..	..	..	..	2,784	1,781	36.0
	H	..	..	..	..	..	..	..	..	285	263	7.7

* Age of males during matings (days): A, 6–7; B, 8–9; C, 10–11; D, 12–13; E, 14–15; F, 16–17; G, 18–19; and H, 20–21.

In O_2, the percentages of translocations in period A, 14.0 per cent, and period B, 15.9 per cent, were not significantly different. In the D and E periods, the percentages increased to 31.6 and 30.9. These values are twice those for the first periods and significantly different from them. There was also a greater number of translocations involving three, four, or five chromosomes in periods D and E than in periods A and B. This may be noted in Table 1 or Table 2, where the percentages of the various types of interchanges were calculated for the different periods. The translocation rate drops in period F, indicating a mixture of meiotic and postmeiotic cells. Neutron treatment in nitrogen shows that translocation damage in nonmotile sperm (3.9 per cent) is significantly lower than that obtained for the remaining mating periods containing postmeiotic cells. The values of 10.0, 11.5, and 9.9

TABLE 2

PERCENTAGE INTERCHANGES INVOLVING TWO, THREE, FOUR, AND FIVE CHROMOSOMES

TEST	PERIOD	NUMBER OF CHROMOSOMES T_2	T_3	T_4	T_5
Oxygen	A	88.0	12.0	..	..
	B	92.6	6.2	1.2	..
	D	79.0	14.4	5.5	1.1
	E	74.5	14.9	8.5	2.1
Nitrogen	A	88.2	11.8	..	..
	B	97.8	2.2	..	..
	D	88.0	8.0	4.0	..
	E	93.4	3.3	3.3	..

per cent for periods B, D, and E showed no significant differences. In N_2, the translocation damage in spermatids (period E) was about twice that for non-motile sperm. In N_2 percentages for interchanges involving two or more chromosomes (Table 2) were not different in the various periods. In period F, translocation damage dropped to 3.0 per cent.

Comparison of translocation damage in the O_2 and N_2 tests shows that consistent and significantly higher percentages of translocations were recovered in all postmeiotic cells with O_2 (Fig. 1). The percentages in O_2 were three times as great as in N_2 in both sperm cells (period A) and spermatids (periods D and E). The difference between translocation damage in the two gases was not so great in period B. Although this discrepancy could be important in regard to sensitivity differences, there is too great a possibility that it results from a mixture of sperm bundles and spermatids of different sensitivities. A better comparison for sperm can be made by using the first samples (period A), which will contain a more consistent sample of cells treated as sperm bundles. Spermatids designated in periods D and E were from samples large enough to separate spermatids from sperm bundles and were separated from meiotic cells by differences in translocation rate.

Spermatids were approximately twice as sensitive as sperm when O_2 was reduced or even eliminated by replacement with N_2 in the neutron treatment. Chromosomes in spermatids are more sensitive to chromosome breakage than those in sperm. An explanation by chromosome restitution would be to assume that, in N_2, restitution of breaks occurs more often in sperm than in spermatids. In O_2, translocation damage in spermatids is also twice that in sperm. In both gases the frequency of translocations in spermatids is about twice that for sperm. These results would not be expected if the gaseous atmospheres had an effect upon the restitution of breaks.

In the O_2 test, the number of spermatid cells that contained translocations increased, and the minimum number of breaks also increased (Table 2). The relative proportion of translocations involving more than two chromosomes increased from 8–12 per cent to 21–25 per cent. This characteristic shift in the number of chromosomes involved shows that increases in translocation damage in spermatids were the result of increases in breakage. Spermatid cells are sampled later in the cycle than sperm cells, and the longer period of time between treatment and sampling does not appear to increase normal healing of breaks.

With O_2, dominant lethals in postmeiotic cells (periods A through E) exhibited a significant increase from 68.7 per cent in sperm to 92.4 per cent in spermatids (Table 1 and Fig. 2). In N_2, the percentages of dominant lethals recovered from

the mating periods A through E (postmeiotic cells) were approximately the same. Absence of a significant increase in dominant lethals in spermatids indicates either a difference in dominant lethals and translocations or differences in sperm release in the N_2 tests. The mating procedure makes it difficult to assume selective mating of certain females in the N_2 test and not in O_2. An increase in the biological damage occurred in period D or E as compared to the first mating period in a series of some 20 tests with various types of radiation and in a number of gas mixtures[1-3]. A lack in proportional increases in lethal damage in spermatids over that for sperm would indicate a qualitative difference in response of the various cells to O_2. Values for lethal damage in N_2 remained similar in postmeiotic cells, whereas, in O_2, the lethal damage increased in varying amounts in different cells (Fig. 2).

Tests for translocation and dominant lethal damage were repeated with O_2 and with N_2 using mature sperm from 7-day-old males. A dose of 1,004 rads of 14-Mev neutrons produced 52.7 per cent dominant lethals in O_2 and 42.1 per cent in N_2. Oxygen was also effective for increasing translocation damage in mature sperm, 9.2 per cent translocations being obtained with O_2 and 3.5 per cent with N_2. Differences in biological damage in O_2 and N_2 atmospheres correspond to those obtained for sperm bundles in Tests I-1 and I-2.

In samples of the germ cells including meiotic stages, period F, smaller percentages of lethals were recovered with both gases. The values approached the control level in the spermatogonial cells of periods G and H. Lethal values remained slightly higher in O_2 than in N_2 in periods F and G. This may indicate an O_2 effect.

The pupae tests (Table 3 and Fig. 3) furnish more information on neutron damage in spermatogonial cells. The neutrons produced maximum damage in the post-

TABLE 3

DOMINANT LETHAL DAMAGE FROM EXPOSURE OF PUPAE TO NEUTRONS

Period	Days of Mating Period	Eggs	Pupae	Per Cent Dominant Lethals	Eggs	Pupae	Per Cent Dominant Lethals
		300 Rads of 14-Mev Neutrons					
		Oxygen (III-1)			Nitrogen (III-2)		
A	3	1,928	0	100.0	65	49	24.6
B	2	1,393	0	100.0	3,516	31*	99.1
C	2	1,159	0	100.0	2,207	35*	98.3
D	2	1,592	0	100.0	1,724	101*	94.2
E	2	2,847	0	100.0	4,591	102*	97.8
F	4	2,772	2,586	6.7	3,702	3,278	11.4
G	2	1,449	1,349	6.9	1,509	1,353	10.3
H	2	3,531	3,275	7.3	2,735	2,540	7.1
I	4	3,717	3,615	2.7	3,296	3,171	3.8
		200 Rads of 3-Mev Neutrons					
		Oxygen (IV-1)			Nitrogen (IV-2)		
A	3	1,023	0	100.0	1,056	0	100.0
B	2	1,772	0	100.0	1,664	0	100.0
C	2	1,564	0	100.0	3,849	0	100.0
D	2	437	1	99.8	391	0	100.0
E	2	1,620	1,504	7.2	3,671	3,313	9.8
F	2	863	764	11.5	1,386	1,259	9.2
G	2	1,267	1,184	6.6	2,523	2,400	4.9
H	4	2,639	2,495	5.5	4,110	3,916	4.7

* All pupae in B, C, D, and E from the same male.

meiotic cells, and analysis for comparative damage in O_2 and N_2 are not possible. The few offspring that were recovered in the N_2 tests were from the same male.

The 300-rad dose of 14-Mev neutrons produced more damage than might be expected by comparison with the 999.5 rads used in the imago tests. In pupae 110–115 hours old, spermatogonia, spermatocytes, spermatids, and a few sperm bundles are present in the testes.[4] The difference in dose rate of 25 rad per hour for the pupae and 2,000 rad per hour for the adult test appears to be the only difference if germ cells are equally sensitive in pupae and imago stages. A slow dose rate over a longer time may eliminate more cells.

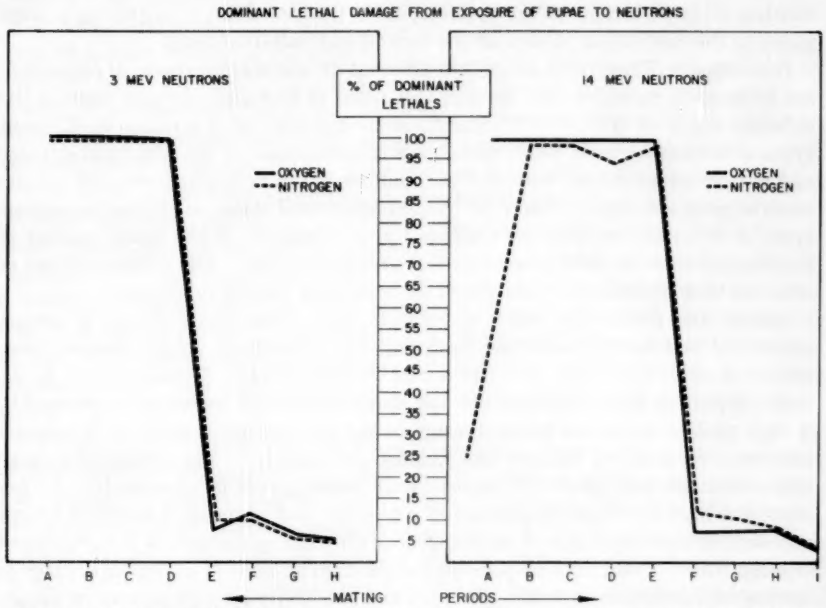


FIG. 3.—Dominant lethal damage from exposure of pupae to accelerator neutrons. Mating periods *E* through *H* for 3-Mev neutrons and periods *F* through *I* for 14-Mev neutrons indicate samples from premeiotic cells.

The high dominant lethal values for postmeiotic cells eliminated samples for translocation tests, and the best estimates for postmeiotic samples must be inferred from X-ray expermients.[1] In these tests, translocations were recovered from the first two mating periods, and period C consisted predominantly of meiotic cells, as indicated by the drop in translocation rate. The mating periods A–E or A–D in the pupae tests with neutron treatment represent postmeiotic and meiotic cells. Spermatogonial or stem cells are indicated by the sudden and distinct drop in dominant lethal damage from 100 per cent to values approximating the control levels. Lethal values similar to the control values were retained in samples taken over a period of 10–12 days. Recovery of fertility by the males shows that some types of cells are resistant enough to survive neutron doses that produce complete lethality in spermatids and meiotic cells. The small values for lethals and the survival of resistant cells do not indicate that biological damage has not been produced in spermatogonial cells. Absence of mature sperm in some periods with fission neutrons in-

dicates that injured cells may be eliminated by cell degeneration.[2] In the two different pupae tests, the lethal damage fell from 100 per cent to near control level, and it would be difficult to assume that this difference could be entirely the result of radiobiological resistance of spermatogonial or stem cells.

An average control value of 94.5 per cent for egg development can be calculated from a total sample of 38,355 eggs and 35,257 pupae. Control tests have been run with adults, young male, and pupae tests. The value for any test or mating period varied no more than 3 per cent from this value. There is, however, a consistent increase in the percentage of egg development in the second day of egg laying as compared to the first day in almost all the control and radiation tests.

Discussion.—When the germ cells of different developmental stages of *Drosophila* are exposed to radiation, the amount and types of biological damage vary in the different types of cells.[1-3, 7-9] Simultaneous exposure of a number of different types of developing germ cells allows a direct comparison of the biological damage and reduces experimental variation from differences in radiation doses and in mixtures of germ cell types. Data for the developmental stages of the spermatogenic cycle of *D. virilis*, reported by Clayton,[4] give a measure of the mean number of histological types of cells present at the irradiation time. The different types of cells can then be separated and tested by controlled mating procedures.

Among the postmeiotic cells, spermatids have consistently shown a greater sensitivity to different radiations, including fission neutrons, accelerator neutrons, 200-kv X-rays, 1.17–1.33-Mev γ-rays and 22-Mev X-rays (betatron).[1-3] In *D. virilis*, a peak in both translocations and dominant lethals appeared in spermatids. A high peak in dominant lethal damage in the spermatogenic cycle of *D. melanogaster* was reported by Bonnier and Lüning[7] and Lüning.[8] The biological damage was correlated with germ cell stages for *D. melanogaster* by Auerbach.[9] In her study the high mutation frequencies of sex-linked and autosomal recessive lethals appeared in the early stages of spermiogenesis after the appearance of X-ray-induced crossing over. Conditions responsible for the high amount of biological damage in spermatids involve the presence of oxygen and sensitivity for chromosome breakage. Increase in biological damage with X-irradiation in O_2 atmospheres was quite pronounced in *D. virilis*,[1] and Sobels[10] found that azide and cyanide with X-rays noticeably increased the mutation rate for similar periods in *D. melanogaster*. Both indicate an involvement of oxygen metabolism in radiation damage. Neutrons as well as X-rays have been shown to produce quantitative differences in the amount of biological damage with changes in environmental conditions of oxygen, temperature, and hydration.[1, 2, 11-15] Neutrons do not produce such pronounced differences in biological damage with environmental changes as do X-rays.[13, 15] In *D. virilis*, the sensitivity difference for spermatids and sperm with fission neutrons and 200-kv X-rays differed by a factor of 2. In air, twice the dose of neutrons was required to produce 50 per cent lethal damage in sperm as in spermatids: with 200-kv X-rays, a dose at least four times as large was necessary. The relative biological effectiveness of fission neutrons and X-rays in sperm cells shows that the doses necessary for 50 per cent lethal damage are 2880 rads for X-rays and 432 rads for neutrons. This gives a relative biological efficiency (RBE) of 6.6. For spermatids an RBE value of 3.2 is obtained from the doses of 750 rads for X-rays and 233 rads for fission neutrons.[2] Data for 14-Mev accelerator neutrons included here and unpublished data

indicate that in sperm the values are closer to 2 or 3. These values are in close agreement with the RBE values obtained for mature sperm of *D. melanogaster* (Edington and Randolph).[5] For spermatids, the RBE values would approach a value half that for sperm if translocation damage was twice as great in spermatids as in sperm with the same dose of neutrons.

Two different radiochemical reactions may be predominant when 14-Mev neutrons and 200-kv X-rays are used. Based on the results of Allen[16] for the decomposition of H_2O by ionizing radiations, irradiated H_2O decomposes to molecular H_2 and H_2O_2 simultaneously with the decomposition to the free radicals H and OH. With γ-rays and hard X-rays, the yield of free radicals is the predominant reaction, and, as the ion density of the radiation increases, the molecular yield increases as the free radical yield falls. According to Bonet-Maury's results,[17] free radical formation is predominant for radiations with ion densities below 200 ion pairs/μ. Above 200, the predominance of the molecular decomposition of H_2O is indicated by the high yields of H_2 and H_2O_2. Formation of free radicals with radiations over 200 ion pairs /μ has been attributed to irregular spacing of radicals along the ion column[16] and to the side tracks and ends of electronic trajectories.[11] With X-rays below 200 ion pairs /μ, the radical distribution would be more sparse and more uniform and allow a re-formation of H_2O or back reactions resulting in a series of reactions involving free radicals. The 14-Mev neutrons, which yield an average ion density of 400 ion pairs /μ, would fall into the predominantly molecular type of reaction. The energy density is not high enough to eliminate free radical formation and should allow testing free radical formation along with the molecular type of reaction.

A direct action from the molecular decomposition type of action should produce parallel values for biological damage in O_2 and N_2 with postmeiotic cells. We found this situation essentially true for translocations. This indicates that chromosome breakage varies in sensitivity during spermiogenesis. In O_2 the percentage of translocations was increased from the N_2 value of 3.9 to 14.0 per cent. In spermatids, the N_2 value of 9.9 per cent was increased to 30.0 per cent. In period B the increase with O_2 was lower than would be expected, but the variability in this period was probably caused by mixtures of sperm and spermatids. An increase in translocation damage in the immature spermatids over the value for mature sperm in N_2 atmospheres indicates a difference in the sensitivity for chromosome breakage. The threefold increase in O_2 above the N_2 value was found for sperm and spermatids. An additional effectiveness from O_2 does not seem to occur in spermatids. The increase with O_2 could be by a direct action, as indicated by P. Alexander,[18] although formation of free radicals is not eliminated by the LET energies for 14-Mev neutrons. A doubling of translocation damage in spermatids both in O_2 and N_2 with accelerator neutrons and in air with fission neutrons necessitates an interpretation that at least a portion of the effect is from breakage differences. Oxygen acts in some direct way to cause an increase in chromosome breakage, since breakage in all postmeiotic types of cells in the O_2 test was higher than in those treated with N_2.

Results for the induction of dominant lethals in the various stages of spermiogenesis were different from those for translocations. Values in N_2 did not show significant increases in spermatids, although an increase was evident in O_2. These results indicate that the cells vary qualitatively in their response to the same O_2

concentration. An explanation by means of differential mating of the males does not seem satisfactory from the method used, and absence of an increase in lethal damage has not been encountered before.

With O_2, X-rays produce greater increases in translocation rates in spermatids than in sperm, whereas the differential in rates in N_2 seems to be smaller and more like the situation obtained with neutrons. The rate in N_2, with over 2,000 mature sperm, has been tested in four experiments. Values of 3.6, 3.9, 4.1, and 5.7 per cent for the various tests agree with the rate reported by Baker and Edington.[19] The best estimate for spermatids in N_2 is from pupae treated with 1,000 r. The translocation rate from the first mating period was 1.6 per cent and was 5.5 per cent for the second period.[1] The rate dropped to less than 1 per cent when meiotic cells were included in the sample. The second mating period is considered more representative of spermatids, since the first period contains sperm bundles. According to Clayton, there is a very short period in germ cell development where the testes contain spermatids without the presence of sperm bundles. On this basis, spermatids are twice as sensitive to chromosome breakage as sperm, since 2,000 r produced the same percentage of translocations in sperm as 1,000 r did in spermatids. Pupae tests are not desirable because of the limited sample and erratic appearance of sperm bundles in the germ cell population at this time. Values of 2.5 and 5.0 per cent translocations were obtained for sperm and spermatids by treating young males with 1000 r of x-rays.

Biological damage recovered from meiotic and spermatogonial cells differs from that obtained for postmeiotic cells. Gross rearrangements have not been recovered from spermatogonial cells in either the *D. virilis* or the *D. melanogaster*[20] tests. This absence of two break rearrangements in meiotic and premeiotic stages has also been reported for *Drosophila* females[21] and *Sciara* females.[22] Another characteristic difference for meiotic and spermatogonial cells is that of cell degeneration. This has not been detected for cells treated in postmeiotic stages. In spermatogonial cells, radiation produces cell degeneration in the testes of the treated males. Such cellular types of lethals can be detected by the reduction or complete absence of mature sperm in males during certain mating periods. Females used for matings during these periods receive few or no sperm from the males, and many of the eggs deposited do not contain sperm. In *D. virilis*, with the mating procedure used, egg samples that contained mixtures of fertilized and unfertilized eggs were obtained in period G.[1] Auerbach[9] reported a reduction in the number of mature sperm available in *D. melanogaster* males during approximately similar mating periods.

With X-radiation, lethal damage appears to be attributable both to the absence of sperm to fertilize eggs and a "genetic lethal" type in which the eggs contain sperm but fail to develop. If the presence of sperm in the egg is considered indicative that development has been initiated, these may be considered similar to the lethal types in postmeiotic cells. The data are not extensive enough to eliminate completely the possibility of mixtures of different types of germ cells. After only 200 r of X-rays, the proportion of unfertilized eggs was not sufficient to account for the total number of eggs that failed to develop.[3] With fission neutrons, complete cell degeneration is indicated in period G. The preceding period showed no noticeable drop in the proportion of fertilized eggs, although the following period

may be affected.[2] Preliminary data for γ-rays and 22-Mev X-rays indicate that cell degeneration of spermatogonial cells also occurs with these radiation.[3]

Pupae data for 14-Mev neutrons show that resistant types of spermatogonial or stem cells are present in the testes. The noticeable drop from complete lethality to control level for lethals indicated that some types of lethal damage in the spermatogonial cells are being selected out or that the difference in the resistance of the cells is quite great. The possibility of selection against lethals in spermatogonial cells is supported by the data for cell degeneration obtained with fission neutrons. The histological data first reported by Friesen[23] and later work of Welshons and Russell[24] show that degeneration of late spermatogenia and early spermatocytes occurs after radiation.

Summary.—Germ cells of spermatogenesis of *D. virilis* were treated with accelerator neutrons of 14-Mev energy in O_2 and N_2 atmospheres. Treatment with 999.5 rads of neutrons in O_2 produced significant increases over the N_2 values for translocations and dominant lethals in postmeiotic stages of cell development. In both gases the differential effect was greater in spermatids than in sperm. In spermatids the influence of environmental changes (presence of O_2) increased the translocation damage from 10 per cent in N_2 to 30 per cent in O_2. The minimum number of breaks also increased, showing that O_2 exerts an effect on sensitivity for chromosome breakage. Dominant lethal damage was also noticeably increased in sperm and spermatids with O_2. In N_2, there were no significant changes in dominant lethal values in postmeiotic cells.

Sensitivity in chromosome breakage, measured as translocations, differed in the germ cell stages in both gases. In N_2, twice the amount of translocation damage was produced in spermatids as in sperm treated with the same dose of neutrons. In the O_2 test, which was run simultaneously with the N_2 test, consistently higher values were obtained, but a similar differential effect was found for sperm and spermatids. This similarity in differential breakage in both gases shows that chromosome breakage differs in sperm and spermatids and that the difference is not limited to external atmospheres of gases that contained O_2, since differences also occurred in N_2. A comparison of dominant lethals in N_2 and O_2 shows qualitative differences in lethal damage in the germ cells of spermiogenesis when O_2 is present. Dominant lethal values for sperm and spermatids were similar with N_2.

In meiotic cells, higher dominant-lethal values were found with O_2 than with N_2. In spermatogonial cells, the lethal damage was similar in both gases. Spermatogonial cells were also obtained from pupal tests and gave similar results in both gases. The pupal test showed that some premeiotic cells were present in the testes resistant enough to survive neutron doses that produced complete lethality in postmeiotic cells. The surviving cells contained no dominant lethal damage and acted as stem or spermatogonial cells for repopulating the germ cells of the testes.

The mean value for the LET of 14-Mev neutrons is higher than that for 200-kv X-rays and lower than that for fission neutrons. The relative biological efficiency falls in an intermediate range between that for X-rays and fission neutrons for postmeiotic cells.

I should like to express my appreciation to Dr. Alexander Hollaender, director of the Biology Division, Oak Ridge National Laboratory, for his interest and support of this project: Dr. M. L. Randolph, of ORNL, for the neutron calibrations

and help on related problems: Dr. Marvin Kastenbaum, of ORNL, for the statistical analyses of the neutron data: and Professors Wilson S. Stone and Charlotte Auerbach for criticism and suggestions on preparation of the manuscript. Appreciation is also due Madeleine Brittain, Janet Bergendahl, Zuelema Johnson, and Margarèt Mazeika for technical assistance.

* This project was supported by the Oak Ridge National Laboratory, operated by Union Carbide Corporation for the U.S. Atomic Energy Commission and USAEC Contract No. AT-(40-1)-1828 with the University of Texas, M. D. Anderson Hospital and Tumor Institute. Support during the course of the investigation was also furnished by the University of Texas, M. D. Anderson Hospital, and the Oak Ridge Institute of Nuclear Studies, Participant Contract No. S-505 under Contract No. AT-(40-1)-Gen-33. A portion of this work was carried out while the author was a research participant in the Biology Division of the Oak Ridge National Laboratory.

[1] M. L. Alexander and W. S. Stone, these PROCEEDINGS, **41**, 1046, 1955.

[2] M. L. Alexander, *Genetics*, **41**, 631, 1956; *ibid.* **43**, 458, 1958.

[3] M. L. Alexander, *ibid.*, **42**, 357, 1957; *Radiation Research*, **9**, 85, 1958; Texas Reports on Biology and Medicine, Radiation Biology and Cancer (in press).

[4] F. E. Clayton, *J. Morphol.*, **101**, 457, 1957.

[5] C. W. Edington and M. L. Randolph, *Genetics* (in press); A. D. Conger, M. L. Randolph, C. W. Sheppard, and H. J. Luipold, *Radiation Research* (in press).

[6] E. F. Oakberg, *Radiation Research*, **2**, 369, 1955.

[7] G. Bonnier and K. G. Lüning, *Hereditas*, **36**, 445, 1950; *ibid.*, **39**, 193, 1953.

[8] K. G. Lüning, *Hereditas*, **40**, 295, 1954.

[9] C. Auerbach, *Z. indukt. Abstammungs- u. Vererbungsl.*, **86**, 113, 1954.

[10] F. H. Sobels, *Z. indukt. Abstammungs- u. Vererbungsl.*, **86**, 399, 1955.

[11] L. Ehrenberg, *Acta Agr. Scand.*, **4**, 365, 1954; L. Ehrenberg and N. Nybom, *Acta Agr. Scand.*, **4**, 396; L. Ehrenberg, A. Gustafsson, U. Lundquist, and N. Nybom, *Hereditas*, **39**, 493, 1953.

[12] W. S. Stone, *Brookhaven Symposia in Biol.*, **8**, 171, 1956.

[13] L. H. Gray, A. D. Conger, M. Ebert, S. Hornsey, and O. C. A. Scott, *Brit. J. Radiol.*, **26**, 638, 1953.

[14] A. Hollaender, W. K. Baker, and E. H. Anderson, *Cold Spring Harbor Symposia Quant. Biol.*, **16**, 315, 1952.

[15] N. H. Giles, Jr., A. V. Beatty, and H. P. Riley, *Genetics*, **37**, 641, 1952.

[16] A. O. Allen, *Discussions Faraday Soc.*, **12**, 79, 1952.

[17] P. Bonet-Maury, *Discussions Faraday Soc.*, **12**, 72, 1952.

[18] P. Alexander, *Radiation Research*, **6**, 653, 1957.

[19] W. K. Baker and C. W. Edington, *Genetics*, **37**, 665, 1952.

[20] C. L. Ward and M. L. Alexander, *Genetics*, **42**, 42, 1957.

[21] B. Glass, *Genetics*, **40**, 252, 281, 1955.

[22] M. L. Bozeman and C. W. Metz, *Genetics*, **34**, 285, 1949.

[23] H. Friesen, *Biol. Zhur.*, **6**, 1055, 1937.

[24] W. J. Welshons and W. L. Russell, these PROCEEDINGS, **43**, 608, 1957.

*THE SOMATIC CHOMOSOMES OF MAN**

By J. H. Tjio and T. T. Puck

ESTACIÓN EXPERIMENTAL DE AULA DEI, ZARAGOZA, SPAIN
AND
DEPARTMENT OF BIOPHYSICS, FLORENCE R. SABIN LABORATORIES,
UNIVERSITY OF COLORADO MEDICAL CENTER, DENVER

Communicated by H. J. Muller, October 6, 1958

Until 1956 the somatic chromosome number of man was generally considered to be $2n = 48$. In that year, however, observations were published that strongly indicated the real somatic number to be 46.[1,2] This chromosome number was later confirmed by several other investigators, whose results are reviewed in a recent paper by Ford, Jacobs, and Lajtha.[3] Since 1956, only Kodani[4] has reported human chromosome numbers other than 46, viz., 47 and 48. Ford, Jacobs, and Lajtha,[3] in reporting the results of their studies on human bone marrow, also summarized all human cases with 46 chromosomes and arrived at a total of 60, excluding those of Kodani. To these can now be added all 13 individuals whose cells were studied in tissue culture at the Department of Biophysics, University of Colorado, Denver, in the autumn of 1957, and one case in which spermatogonial mitosis and meiosis were also examined. Thus the total number of individuals found to possess 46 chromosomes now stands at 74.

The present paper deals with the analysis of the human karyotype of normal tissue cells grown in vitro by the methods previously described. In Table 1 the

TABLE 1

Chromosome Counts of Human Cells Cultivated in Vitro

			No. of Cells Counted Exactly			
			Diploid Cells			Polyploid Cells*
Case	Sex	Organ of Origin of Biopsy	45	46	47	92
1....	M	Skin	1	352	..	27
2....	M	Skin		35	..	1
3....	M	Lung		75	..	2
4....	M	Prepuce		112	..	5
5....	M	Testes		105	..	6
6....	F	Cervix		314	1	28
7....	F	Cervix		172	..	10
8....	F	Myo- and endometrium		138	..	6
9....	F	Ovary		112	..	7
10....	F	Skin		65	..	2
11....	F	Skin		103	..	11
12....	F	Skin		110	..	3
13....	F	Skin		24	..	
Total			1	1,717	1	108

* Less than 3 per cent of the mitotic figures were polyploid. Hence polyploid cell counts were performed on larger cell samples than those from which the diploid mitoses arose, in order to provide a number of polyploid cells sufficiently large to be reliable. As the table shows, in the sample of 108 polyploid cells selected at random, no chromosome number other than 92 was encountered.

cultures are listed together with the organs from which they originated and the number of cells on which exact chromosome counts were made. As can be seen from the table, the cells were taken from several different organs. The organ of origin of the initial biopsy had no influence on the chromosome numbers or morphologies. The ages of the subjects varied from one to forty-one years. All cells

were of a fibroblast-like morphology and were grown as previously described in a synthetic nutrient medium, supplemented with 15 per cent fetal calf serum which was pretested with respect to its efficiency in promoting colony formation from single mammalian cells[5]. The cell strains were cultivated as monolayers on glass, and exhibited a doubling time of 16–24 hours. Cells were harvested by trypsini-

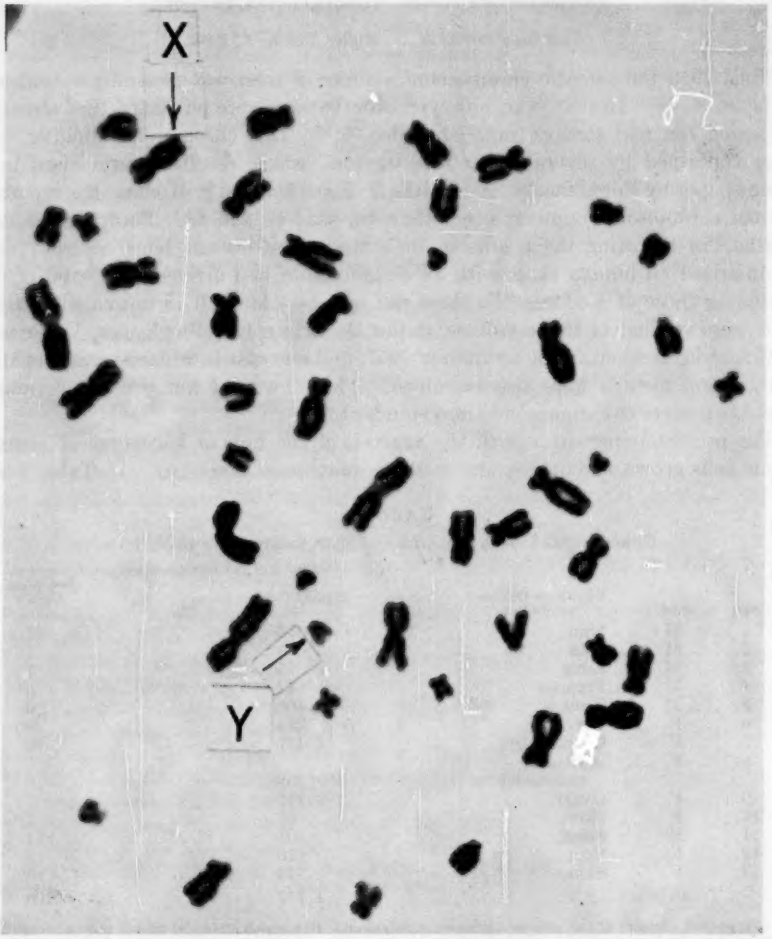


FIG. 1.—Photomicrograph of a colchicine metaphase of a human male cell grown in vitro. The biopsy was obtained from the skin of an adult. ×2,000.

zation once or twice a week. The temperature, pH, and per cent of CO_2 and water in the gaseous phase were regulated most carefully during the growth of the cells. Cells multiplying under these controlled conditions for as much as 8 months, during which more than 70 generations of growth occurred (equivalent to more than 10^{21} progeny), have shown no variation in chromosome number or morphology,

except for a small percentage of polyploid cells. Chromosome examination of these cells was carried out on slides prepared in accordance with the technique reported earlier.[6]

The data of Table 1 indicate the constancy of chromosome number obtained

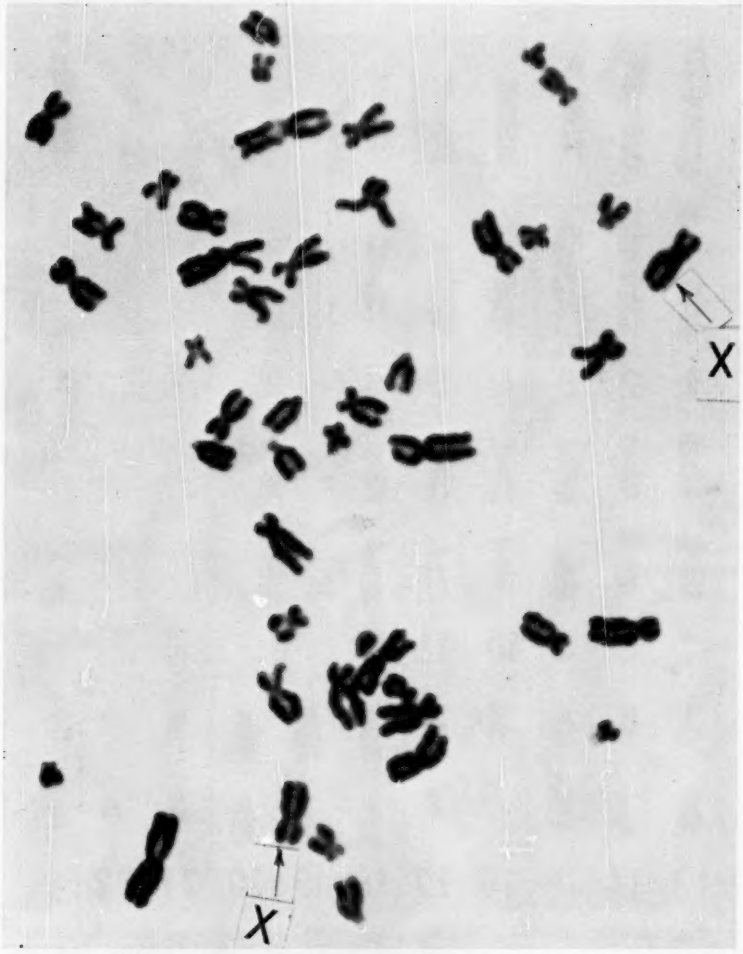


FIG. 2.—Photomicrograph of a human female cell in colchicine metaphase grown in vitro. The biopsy was taken from the cervix of an adult. ×2,000.

from such cells. The two cells with 45 and 47 chromosomes, respectively, might be due either to mitotic non-disjunction or to technical accidents. The number of tetraploid cells in Table 1 does not represent the true polyploid incidence in our material but is simply a record of the exact counts performed on approximately 100 cells with polyploid constitution, all of which turned out to be exactly tetra-

ploid. The total frequency of polyploidy was scored on about 1000 cells in prophase and metaphase and was never higher than 3 per cent.

The quality of the slides can be judged from the photographs of Figures 1 and 2. The idiograms given in Figures 3 and 4 are constructed from such cells. The chromosomes have been cut out and arranged as pairs according to their morpho-

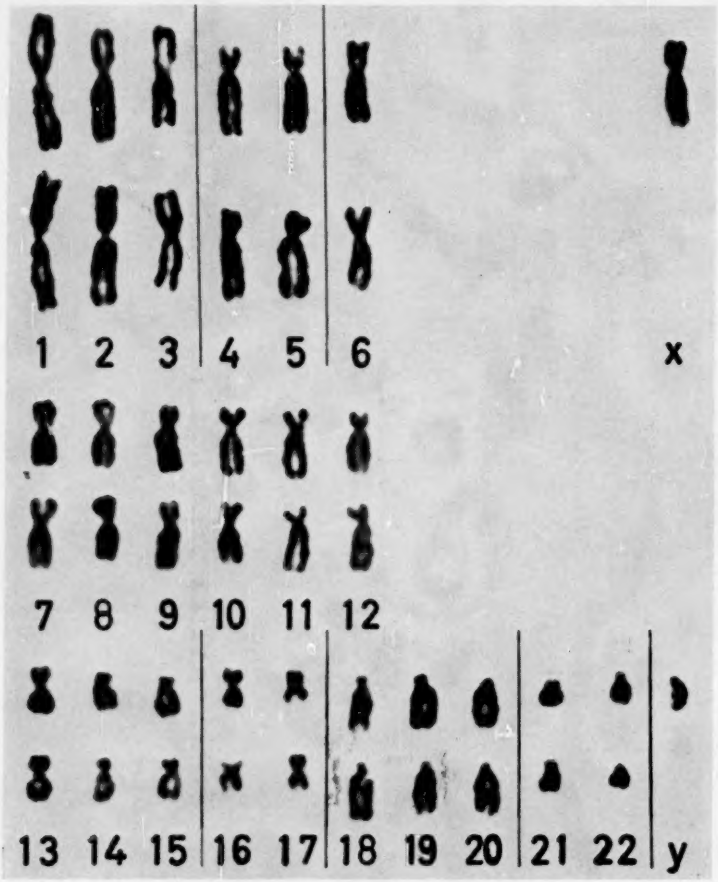


FIG. 3.—The idiogram of a human male. The chromosomes are from an enlarged photomicrographic print (original enlargement 4,000×) cut singly, paired, and arranged in rows and groups for easier identification of the different types. The numbers and groups are the same as in Table 2.

logical characteristics. The pairs have been assembled in 8 groups on the basis of their sizes and the positions of their centromeres (Table 2).

Table 2 is based on measurements from 4 male and 4 female cells. The mean length of the individual chromosome types is expressed as the per cent of the total mean haploid autosomal complement of these cells. The X and Y chromosomes

have been excluded from the total haploid complement, in order that a fixed standard of reference may be employed for both sexes. The data from male and female autosomes have been pooled, as there were no appreciable differences either in arm ratio or in the per cent of the total haploid autosomal length between the corresponding chromosome types of male and female cells in the human material

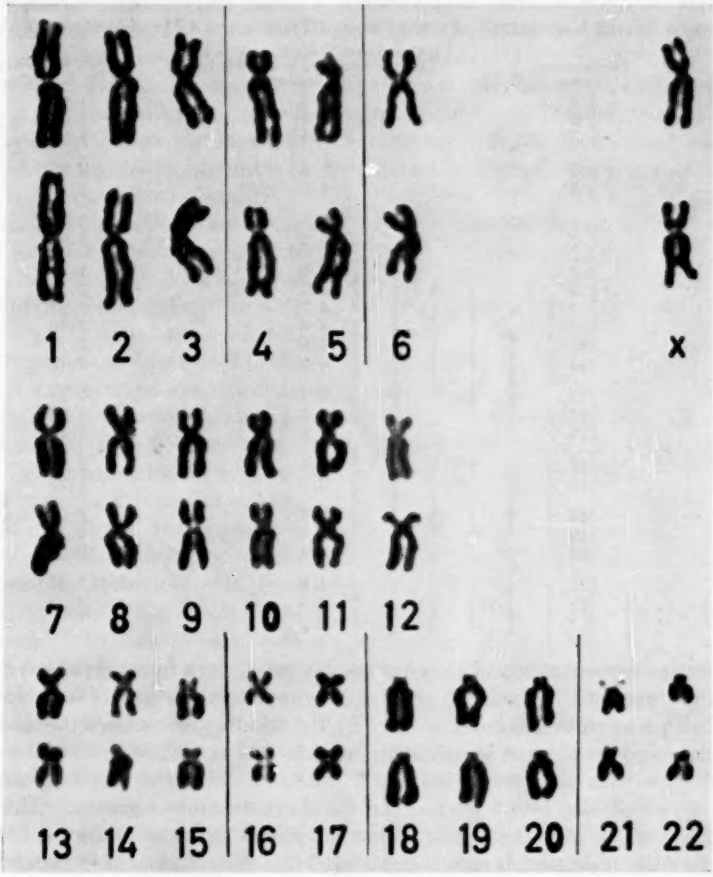


FIG. 4.—The idiogram of a human female. The chromosomes are from an enlarged photomicrographic print (original enlargement 4,000×) cut singly, paired, and arranged in rows and groups as in Table 2.

studied by us. In this respect our observations are different from those carried out on the Rhesus monkey by Rothfels and Siminovitch,[7] who found that the mean arm ratio of the chromosomes is generally higher in female nuclei than in the male. Measurements of the chromosomes were carried out on enlarged photographic prints magnified about 4,000 times. In a series of measurements the length of the longest pair of chromosomes was found to be 6.8 $\pm 1.4\ \mu$, while that of the shortest

pair was 1.36 ± .31 μ. However, considerable variation from these absolute lengths (though not their ratio) may be obtained in different preparations depending on the degree of chromosome contraction exhibited by the individual cells when slides are prepared according to the technique here employed. The diagram of Figure 5 is based on the data given in Table 2.

TABLE 2

ANALYSIS OF HUMAN KARYOTYPE: AVERAGE FROM 4 FEMALE AND 4 MALE METAPHASE PLATES

Group	Chromosome Type	Per Cent Index Mean Length = 100× Length/Total Autosomal Haploid Complement	Ratio Arms Long/Short
I	1	9.6	1.08
	2	8.7	1.56
	3	7.4	1.20
II	4	6.8	2.85
	5	6.2	3.18
III	6	5.8	1.69
	X	6.3	1.94
IV	7	5.0	1.31
	8	4.7	1.50
	9	4.7	1.92
	10	4.6	2.40
	11	4.6	2.78
	12	4.5	3.13
V	13	3.4	1.78
	14	3.1	2.83
	15	2.6	3.75
VI	16	2.3	1.43
	17	2.2	1.29
VII	18	3.7	8.00 (satellited)
	19	3.4	7.33
	20	3.1	10.50
VIII	21	1.9	3.67 (satellited)
	22	1.8	3.33
	Y	2.0	..

For easier indentification of the chromosome pairs, the various types have been placed in three rows in Figures 3 and 4. The upper row consists of long chromosomes with a mean length index of 9.6–5.8; the middle row contains the medium-sized chromosomes of mean length index 5.0–4.5; and in the last row are the small chromosomes with mean length index of 3.7–1.8 (see Table 2). The long chromosomes are subdivided into 3 groups, and the short ones into 4 groups. Although recognition of the individual pairs within the groups in many mitoses is difficult or occasionally uncertain, it should be stressed that the assignment of the chromosomes to their respective groups can always be made with confidence in carefully prepared slides.

As can be seen from the figures and the table, many of these chromosome pairs, such as *1*, *2*, and *3* of group I, can be easily identified. Some of the pairs are more difficult to differentiate from one another, as is the case with numbers *4* and *5* of group II and many of the pairs in group IV. In this latter group the position of the centromere is the most important differentiating characteristic, the ratio of the arm lengths varying progressively from a submedian condition in number *7* to subterminal in number *12*. Pair number *13* of group V is easily distinguishable

by virtue of its more centrally located centromere and larger size, from the pairs *14* and *15*, which are more similar to each other. Pairs *16* an *17* of group VI resemble each other fairly closely, although there is a distinguishing difference in their arm ratios. All the chromosomes of group VII are subterminal. While pairs *18* and *19* of group VII are very similar in length and arm ratios, they are distinguishable by virtue of the satellite possessed by pair number *18*, which is readily recognizable in clear plates. The two small chromosome pairs of group VIII both have a subterminal centromere and are similar to one another, but, again, one of them possesses a characterizing satellite.

Pairing of the chromosomes according to their morphology makes it evident that two chromosomes in the male lack morphologically identical partners. These are obviously the sex chromosomes. Comparison with the chromosome complement of the female demonstrates that the larger of these chromosomes is the X. The Y chromosome is similar in size to the two smallest autosome pairs, and for this reason it has been placed in group VIII of Table 2. It differs from pairs *21* and *22* in having a terminal or almost terminal centromere, as opposed to the distinct short arm possessed by the two autosome pairs of this group. Furthermore, the Y chromosome has a heterochromatic region close to the centric end. In Table 2 the arm ratio of the Y chromosome is left undefined, as it is still uncertain whether this chromosome is telocentric or possesses an exceedingly small arm. In some slides dark bodies are observed close to the centric end which might conceivably be evidence of an extremely short second arm, although they are probably part of the heterochromatic regions which are commonly found close to the centromere in various living forms.

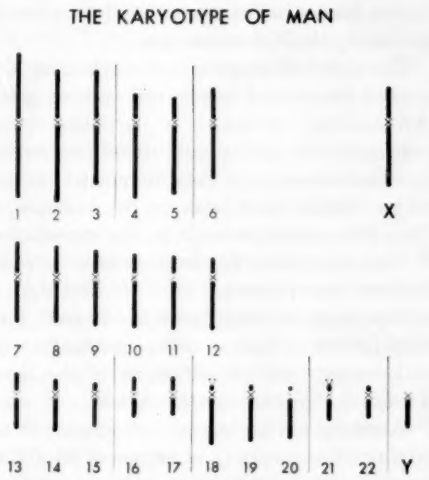


FIG. 5.—Diagram of the human karyotype, set up with data from Table 2.

The X chromosome is one of the largest chromosomes. It is rather similar to the chromosome pair number 6, for which reason it has been placed with this pair in group III. It differs from pair number 6 in being slightly larger and in having its centromere in a somewhat less median position. (In the preliminary report on the morphology of the human chromosomes given in our earlier paper,[6] a tentative opinion was expressed concerning the morphology of the X chromosome, which has now been changed as a result of more detailed analysis presented here.) Without analysis of the whole karyotype, the X chromosome is difficult to identify, especially in female cells because of its similarity with pair 6. On the contrary, the Y chromosome is readily recognized. Identification of the sex of the subject from which the tissue has been taken is thus simple: In males there are 5 chromo-

somes in group VIII, while females have only 4, so that recognition is unequivocal, as shown in Figures 3 and 4.

As already mentioned, there are two chromosome pairs carrying satellites, *viz.*, pairs 18 and 21. These satellites are visible only in particularly clearly delineated preparations. When they are visible, however, they usually are similar in size. Three exceptions to this rule have been found: In the cells of these persons a heterozygosity in the size of the satellites was observed. In one of these subjects there were two heterozygous chromosome pairs, numbers 18 and 21, and in the two others only number 21 was heterozygous.[6] Variations in the size of the satellites, including the occurrence of heterozygous individuals, has been reported in various species of plants.[8-11] The effect, if any, of this stable cytogenetic marker on the human phenotype remains to be studied.

The present observations on the morphology of the human chromosomes are generally in good agreement with the results of Ford, Jacobs, and Lajtha.[3] The main advance in the present study appears to be that it has now been possible also to identify the X chromosome.

The threefold larger size of the human X chromosome over that of the Y, which renders the human female cell approximately 4 per cent greater than the male in chromosomal volume, is of particular interest. This condition confers, at least potentially, a substantially richer genetic capacity on the female, a feature which may underlie some of the differences between the sexes which have been considered to be largely functional, as for example, their notable difference in longevity. This difference also leads to the expectation that different kinds of responses to X-irradiation may be demonstrable in cells from the two sexes in appropriately designed experiments. It is obvious that a knowledge of the karyotype of man is important in many types of research, including an understanding of the action of radiation on human cells; localization of specific human genes; the nature of malignancy; and identification of chromosomal sex of individuals with varying degrees of clinical hermaphroditism.

Summary.—The human chromosomes have been analyzed from cells which originated in a variety of organs of 13 different individuals and which were grown *in vitro* under carefully controlled conditions. Exact counts were performed on almost 2,000 cells, of which 99.9 per cent revealed chromosome numbers of 46 or 92, the tetraploid frequency being less than 3 per cent. The individual chromosomes have been characterized on the basis of their size, position of the centromere, and possession of a satellite and have been assigned to 8 groups. The sex chromosomes have been identified and the X shown to be approximately 3 times larger than the Y.

* Contribution No. 78. This work was aided by a grant from the Rockefeller Foundation and the Commonwealth Fund. These data were presented at the X International Congress of Genetics at Montreal in August, 1958. At this same congress a paper describing aspects of the human karyotype was simultaneously given by Dr. Ernest H. Y. Chu, of Yale University.

[1] J. H. Tjio and A. Levan, *Hereditas*, **42**, 1–6, 1956.
[2] C. E. Ford and J. E. Hamerton, *Nature*, **178**, 1020–1023, 1956.
[3] C. E. Ford, P. A. Jacobs, and L. G. Lajtha, *Nature*, **181**, 1565–1568, 1958.
[4] M. Kodani, these PROCEEDINGS, **43**, 285–292, 1957; *Science*, **127**, 1339–1340, 1958.
[5] T. T. Puck, S. J. Cieciura, and A. Robinson, *J. Exptl. Med.*, **108**, 945–957, 1957.
[6] J. H. Tjio and T. T. Puck, *J. Exptl. Med.*, **108**, 259–268, 1958.

[7] K. H. Rothfels and L. Siminovitch, *Stain Technol.*, **33**, 73–77, 1958.
[8] A. Fernándes, *Bol. Soc. Broter*, II Ser., **10**, 249–277, 1935.
[9] D. Sato, *Cytologia*, **10**, 127–157, 1939.
[10] J. H. Tjio and A. Levan, *Anales estac. exptl. Aula Dei*, **4**, 185, 1956.
[11] S. Bose, *Hereditas*, **43**, 621–643, 1957.

---

*COMPLEMENTATION AT THE AD-4 LOCUS IN NEUROSPORA CRASSA**

BY DOW O. WOODWARD, C. W. H. PARTRIDGE, AND NORMAN H. GILES

DEPARTMENT OF BOTANY, JOSIAH WILLARD GIBBS RESEARCH LABORATORIES, YALE UNIVERSITY, NEW HAVEN, CONNECTICUT

Communicated by Edmund W. Sinnott, October 15, 1958

Three cases of complementation between allelic mutants in heterocaryons of *Neurospora crassa* have been reported in which the individual homocaryons lack a specific enzymatic activity.[1–3] More recent and apparently similar phenomena have been observed in which data relating to the involvement of a single enzyme are still lacking.[4, 5]

The present paper presents a detailed analysis of heterocaryon complementation between *ad-4* mutants, each of which has impaired adenylosuccinase activity. The results suggest that the linear structure of both a gene and its products may be revealed by the pattern of interallelic heterocaryon complementation. The enzyme adenylosuccinase, found in wild-type extracts, catalyzes the splitting of adenosine monophosphate succinate (AMP-S) to adenosine monophosphate (AMP) and fumarate, as well as the splitting of the analogous purine precursor SAICAR to AICAR[6] and fumarate.[1, 7] Adenylosuccinase activity has not been found in appreciable amounts (less than 1 per cent of wild-type activity) in any of the *ad-4* mutants tested, and partial restoration of activity has been detected in all cases examined in which complementation occurs. The degree of complementation varies widely among the different mutant combinations; i. e., growth rates of the heterocaryons on minimal medium range from less than 0.2 to 4 mm/hr (wild-type rate), and enzyme activities range from less than 1 to 25 per cent of wild-type activity. Brief reports of certain of these results have been presented previously.[7, 8]

TABLE 1

CLASSIFICATION OF *ad-4* MUTANTS BY ORIGIN

Strain Used in Obtaining Mutants	Derivation of Strain	No. of Mutants Obtained	Mutagen Used	No. of Complementing Mutants	Mutant Designation
74A		2	None	0	
74A		7	U.V.	1	F31*
74A		19	X-ray	5	F1, F4, F5, F12, F14
5.5A	74A × 73a	1	None	1	F23
5.5A	74A × 73a	3	X-ray	0	
3.1a	74A × 73a	1	None	0	
Y191-R28	F12 revertant	13	X-ray	8	M2-M15
Y191-R13	F12 revertant	7	None	5	M71-M77
Y191-R13	F12 revertant	45	X-ray	21	M27-M70
Y201-R22	F2 revertant	25	X-ray	10	M16-M25
Totals		123		51	

* F mutants listed include only complementing mutants.

Materials and Methods.—The adenine-requiring mutants described in this paper were isolated by the filtration and selective plating technique of Woodward *et al.*[9] The original mutants were derived from the St. Lawrence wild-type strain 74A; other primary mutants were derived from strains 5.5A and 3.1a, which originated from inbreeding of 74A after being crossed with strain 73a. Secondary mutants were obtained from revertant strains Y191-R28, Y191-R13, and Y201-R22. The revertant strains arose by mutation and subsequent reversion in strain 74A at the *ad-4* locus (see Table 1). Mutants were obtained from conidia, either without treatment or following ultraviolet or X-irradiation.

Classification of these mutants into the *ad-4* category is based on their failure to

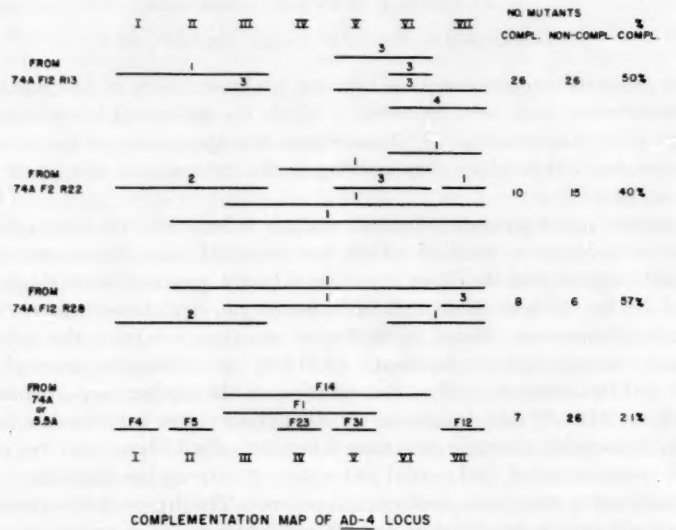


FIG. 1.—Complementation map of the *ad-4* locus based on both primary and secondary mutants. Primary mutants (F designation) are listed individually, since no two have identical complementation patterns. Secondary mutants (upper 3 rows) are listed in groups with numbers above each line referring to the number of mutants exhibiting a particular pattern of complementation. Roman numerals indicate the seven distinguishable functional regions (cistrons).

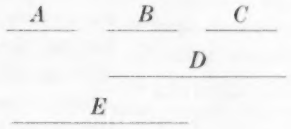
grow on minimal or hypoxanthine-supplemented medium, accumulation of a weak purple pigment when grown on limiting adenine, and their inability to complement with *ad-4* testers (those mutants that do not complement with other *ad-4* mutants). In addition, genetic tests have been carried out on 20 of these mutants, confirming their *ad-4* classification in all cases.[10]

Of 25 mutants assayed for adenylosuccinase activity, no more than 1 per cent of wild-type activity was detected in any mutant extract, and only those with no detectable growth on minimal medium were used in the complementation studies. Assays for adenylosuccinase activity were carried out by following the change in absorption at 280 mμ on a recording spectrophotometer as the reaction proceeds

from AMP-S to AMP. Enzyme extractions were prepared as described in an earlier report[1] except that tris HCl buffer was used in these experiments.

Heterocaryon tests were made by mixing conidial suspensions of two strains on minimal agar Petri plates, as described by de Serres.[11] More comprehensive observations have led to the demonstration of complementation between certain pairs of strains previously reported as negative on the basis of tests of short duration.[1]

Results.—Of the 123 *ad-4* mutants that have been isolated, 51 complement in a heterocaryon with at least one other allelic mutant. Such complementation results in adenine-independent growth of the heterocaryon and partial restoration of enzyme activity. The pattern that emerges when pairwise combinations are tested for complementation can best be illustrated in diagrammatic form (Fig. 1). Sixteen different complementation types are observed among the 51 mutants; the relationships of these mutants indicate the existence of seven distinguishable functional regions (cistrons) arranged in a linear order to form what may be designated a *complementation map.* The actual ordering of mutants on this map is based on mutants having overlapping non-functional regions. Non-functional regions are indicated by solid lines in Figure 1. For example, the ordering of three hypothetical cistrons A, B, and C could be deduced by obtaining mutants (D) which complement only with A (thus overlapping B and C on the map) and others (E) which complement only with C (overlapping A and B). Mutants A, B, and C all complement with each other, while mutants D and E provide evidence for linearity in either the order ABC or CBA:



The entire complementation map of the *ad-4* locus has been deduced by this method. Any overlap in the complementation map is indicative of a lack of complementation within a period of 10 days (see Table 2). In some cases, heterocaryons were established by utilizing nutritional mutant markers; this procedure gave results that were no different from those obtained without the use of such "forcing" markers.[1]

It seems significant that the most widely separated alleles on the complementation map form heterocaryons most rapidly and have, in general, the highest adenylosuccinase activities as measured from crude extracts. Heterocaryons between alleles separated by 2–5 functional cistrons produce up to 25 per cent of wild-type activity (Fig. 2). If alleles are separated by less than 2 functional cistrons, there is a decrease in enzyme activity down to less than 1 per cent of wild type for some combinations involving adjacent-cistron damage. Furthermore, adjacent-cistron combinations generally have the slowest growth rates on minimal medium and require, on the average, a longer time for growth to commence from mixed conidial inocula on minimal agar (Table 2).

Data from secondary mutants derived from F12 revertants (M2-M15 and M27-M77) have not been included in these tables. Complementation data and reverse

TABLE 2*

CLASSIFICATION OF MUTANT COMBINATIONS ON THE BASIS OF TIME REQUIRED TO GIVE A POSITIVE GROWTH RESPONSE AT 25° C.

LOCATION OF DAMAGED SITES ON COMPLEMENTATION MAP	NUMBER OF DAYS AFTER MIXING CONIDIA BEFORE GROWTH COMMENCES									
	2	3	4	5	6	7	8	9	10	More than 10
Adjacent cistrons	16	8	5	3	3	2	0	0	1	2
1-cistron separation	16	3	2	1						
2-cistron separation	15	1								
3-cistron separation	17									
4-cistron separation	13									
5-cistron separation	5									

* Numbers in the body of the table indicate the number of separate heterocaryon combinations of a given class which start growing within the time indicated. Replicate experiments with any given heterocaryon agree within 1 day in nearly all cases (see text).

mutation experiments indicate that a restriction was imposed on F12 at the time of its mutational origin. This restriction prevents complete restoration of enzyme activity by reverse mutation and limits enzyme restoration by complementation. Among a total of 68 F12 revertants assayed, none was found with enzyme activity equivalent to wild type. On the other hand, four other *ad-4* alleles have all given rise to revertants that are quantitatively equivalent to wild type. The revertants from F12 used in obtaining secondary mutants were derived from a category having the lowest adenylosuccinase activity (3 per cent of wild type). There is also evidence of a second restriction's having occurred at the reversion step. Two secondarily derived mutants were found to yield only revertants with activities limited to 3 per cent of wild type from a total of 21 examined. In addition, no heterocaryon involving any two such secondarily derived mutants has manifested more than 3 per cent of wild-type enzyme activity, even when the two damaged sites are separated by 3 functional cistrons on the complementation map. Such a restriction is not observed when mutants of secondary origin complement with mutants derived directly from 74A. However, the use of secondarily derived mutants makes it possible to infer on the basis of the complementation pattern a restoration of the original F12 damage, presumably at the reversion step, and subsequent damage in other cistrons.

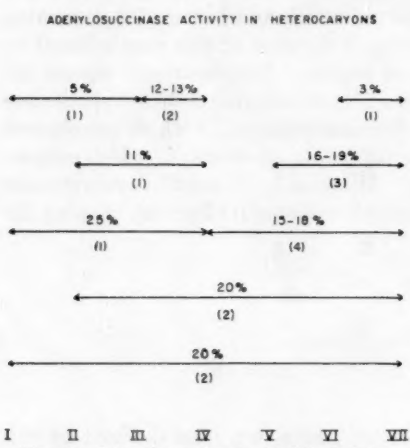


FIG. 2.—Adenylosuccinase activities in heterocaryons expressed as per cent of wild type. Figures below each line indicate the number of different mutant combinations assayed, all of which are separated by the same distance on the complementation map. Roman numerals indicate the seven functional regions.

Certain mutant combinations form heterocaryons that are temperature-sensitive to varying degrees. All temperature-sensitive heterocaryons involve mutants whose combined damage includes the four cistrons, III, IV, V, and VII. Those combinations that result in temperature-sensitive heterocaryons are listed in Table

TABLE 3

GROWTH RATES IN MM/HR OVER 99-HOUR PERIOD ON MINIMAL + HISTIDINE AGAR

Strains	Rate at 25°	Rate at 35°	Blocked Cistrons
F15	3.26	0.10	III IV V VI
F14-M4 heterocaryon	2.14	0.05	III IV V VI VII
F1-F12 heterocaryon	2.95	0.70	III IV V VII
F14-F12 heterocaryon	0.62	0.05	III IV V VI VII
F14-M14 heterocaryon	0.94	0.58	III IV V VI VII
F14-M9 heterocaryon	2.17	1.10	III IV V VI VII
F1-M14 heterocaryon	3.65	2.86	III IV V VII
74A wild type	3.53	3.62	

3, which shows comparative growth rates at 25° and 35° C. F15, a temperature-sensitive mutant, is also listed.

A final and very pertinent question pertains to the linear order as determined by genetic mapping of the locus as compared to the order deduced from the complementation map. Unfortunately, a complete recombinational analysis has not yet been possible because of poor fertility in crosses between *ad-4* mutants. However, the data obtained thus far indicate that certain alleles, widely separated on the complementation map, are also the most widely separated, as judged by the frequency of prototroph recovery from crosses between them.[10]

Discussion.—Perhaps the most striking feature of complementation between allelic mutants is the possibility of describing their relationships in terms of a linear complementation map. A reasonable assumption appears to be that such a map reflects a linear organization of the gene and gene products. On the basis of this assumption and utilizing the quantitative data on adenylosuccinase activity in the heterocaryons, a working hypothesis has been devised to explain the mechanism of complementation at the *ad-4* locus. Nuclear recombination has not been detected in these *Neurospora* strains during vegetative growth, and this hypothesis involves the assumption that interaction occurs between gene products in the cytoplasm.

A critical feature of this hypothesis is the observation that no *ad-4* interallelic (intralocal) heterocaryon has yielded more than 25 per cent of wild-type activity. A maximum of 25 per cent of wild-type activity was also reported for glutamic dehydrogenase from heterocaryons between *Neurospora am* mutants.[2] If some type of cytoplasmic recombination occurred at the RNA or polypeptide level, functional enzyme could be formed by exchanges resulting in the union of two non-defective parts of two gene products damaged at different sites. The reciprocal product of such an exchange would carry both damaged sites. If this type of recombinational mechanism is operative and exchange is random, only one of the four possible combinations would result in the formation of a functional enzyme. The yield of 25 per cent of wild-type activity can be explained by such a mechanism, assuming that a nuclear ratio of 1:1 is approximated in the heterocaryon and also that the sum of normal plus defective enzyme formed in a heterocaryon between *ad-4* mutants is equivalent to the total normal enzyme formed in wild type.

Recent reports have revealed the possibility that recombination at the polypeptide level may indeed occur in some in vitro systems. In the case of ribonuclease[12] it has been found that under certain conditions the modified enzyme dissociates into two fragments—a small peptide and a large component—both of which alone are inactive but which, when mixed under the appropriate conditions, give

restored enzyme activity. Dissociation and recombination has also been reported in mixtures of CO-hemoglobin A and ferrihemoglobin A.[13] This recombination results in the detection of an additional component of intermediate electrophoretic mobility. Furthermore, in a recent study of human haptoglobins, Bearn and Franklin[14] showed the presence of a haptoglobin in heterozygotes which differed from haptoglobins formed by either homozygote in having a peak of intermediate sedimentation rate as well as intermediate electrophoretic mobility, in addition to the peaks characteristic of both homozygotes.

Additional support for some type of random exchange mechanism is furnished by the observation that the time required for growth to commence from conidial mixtures between two mutants can be modified within certain limits by the conidial concentrations used. The conidial concentration should influence directly the relative number of fusions between the two conidial types. A small conidial population size would, on the basis of random exchange between gene products, be less likely to start forming functional enzyme in a given time because of a decrease in the total number of possibilities for exchange. The tendency for heterocaryons between mutants damaged in adjacent cistrons to have less enzyme activity than those between mutant combinations more widely separated on the complementation map could be explained by the same type of restriction as that encountered when linkage distances between mutant loci are less than 50 crossover units. In the case of heterocaryons, this would mean that when damage in two different mutants becomes close enough in terms of position within the locus, the probability of exchange is so low that not enough functional enzyme is formed, compared to the degradation rate, to give a growth response in a given mixed conidial population. Occasionally, in the weakest heterocaryons between *ad-4* mutants (those requiring 6 or more days for growth to commence), growth is delayed more than usual or never commences at all, depending on the conidial concentration. Such sporadic results are never observed in the stronger heterocaryons and would not be expected on the basis of the increased probability for exchange between more widely separated defective sites. The theoretical probabilities for exchange dependent on distance appear to be a reasonable explanation for the quantitative correlation between distance on the complementation map and enzyme activity or growth measurements observed in heterocaryons.

Earlier observations indicated that a heterocaryon between F12 and a nonallelic mutant (an interlocal heterocaryon) yielded 50 per cent of wild-type adenylosuccinase activity.[1] This result can be explained most simply on the assumption that such heterocaryons contain only half as many functional genes for a given locus as does wild type. This type of complementation would not require cytoplasmic interaction as has been suggested for interallelic complementation, but if such interaction occurs, the recombination hypothesis would still predict a 50 per cent yield.

Other hypotheses have been considered as possible mechanisms to account for the partial restoration of enzyme activity in heterocaryons between mutants which lack as homocaryons the same enzyme activity. For example, one nucleus might make all the functional enzyme, in effect receiving from the other nucleus some type of activator. Another possibility is that the product of one nucleus may compete so strongly for an inhibitor of the modified enzyme formed by the other nucleus as

to render the latter capable of carrying out its catalytic function. Evidence is still lacking to indicate definitively whether the enzyme in complementing interallelic heterocaryons is, in fact, a hybrid derived in part from both nuclei or whether one nuclear type produces all the functional enzyme.

More than 50 per cent of the *ad-4* mutants obtained to date fail to complement with any other *ad-4* mutants. On the complementation map these could be represented by a solid bar across the entire map and interpreted as having been damaged in all seven cistrons of the locus. If this were true, however, they would not be expected to occur with such a high frequency. The probability that these are deletion-type mutations for the whole locus is very low, since many of these mutants revert to adenine-independence at a high rate. The unexpected finding that more than twice as many secondary mutants (those derived from F12 revertants) complement with other *ad-4* mutants as do primary mutants may indicate either that a certain amount of non-specific damage or that a specific type of damage is necessary to render a mutant capable of complementation. Secondary mutants have been subjected to three separate dosages of X-ray irradiation (36,000 r each) and have been altered at the *ad-4* locus on each occasion, while primary mutants received only one such dose in their production. The complete absence of interallelic complementation found in some mutants is not irreversible. The primary mutant F2 does not complement with any other *ad-4* mutant, but 40 per cent of the secondary mutants derived from a back mutant of F2 do complement in at least one combination. Furthermore, the over-all pattern exhibited by this group of secondary mutants extends across the entire complementation map (see Fig. 1).

Mutants that do complement but have more than one non-functional cistron cannot easily be visualized as having been damaged in more than one site, since they also occur quite frequently and in most cases are capable of undergoing reversion with restoration of enzyme activity. In such cases of apparent widespread damage within the locus, a single critical site within one cistron may be damaged, resulting in a type of position effect extending over several cistrons. There is evidence from recombination data at the *pan-2* locus that genetic localization is possible even with mutants blocked in several cistrons.[15]

The data from temperature-sensitive heterocaryons indicate that these mutants have damage in common and support the idea that the complementation map is a valid diagrammatic representation of the *ad-4* locus. If the enzymes from such heterocaryons prove to be thermolabile, the data would suggest a direct correlation between the map and the enzyme molecule.

Summary.—One hundred and twenty-three mutants at the *ad-4* locus have been tested for complementation in heterocaryons. Fifty-one of the mutants gave at least one positive response. Although these mutants lack adenylosuccinase activity, partial enzyme activity is restored when complementing mutants are combined in heterocaryons. The relationships of the complementing mutants at the *ad-4* locus are such that these mutants can be arranged in a unique linear sequence which may be designated a complementation map of this locus. This arrangement is possible because of the apparent presence in certain alleles of continuous multiple non-functional regions which overlap non-functional regions of other alleles. The complementation map of the *ad-4* locus is divided into seven functional regions (cistrons). There is a marked correlation between distance on the complementation

map and the strength of the heterocaryon as judged by enzyme activity, growth rate, or time required for growth to commence from mixed conidial inocula. Thus heterocaryons between mutants damaged in adjacent cistrons have low adenylosuccinase activities, and activities increase with increasing distance to the maximum value of 25 per cent of wild type for mutants separated by 2–5 cistrons.

Possible interpretations of these results are discussed and the hypothesis is proposed that interallelic complementation involves the formation of defective gene products by different mutant nuclei in a heterocaryon. Cytoplasmic recombination of such gene products either at the primary (RNA) or secondary (polypeptide) level, perhaps by a process analogous to crossing over, could yield enzymatically active protein.

The authors would like to acknowledge the co-operation of Dr. Mary Case and the technical assistance of Mrs. Dow O. Woodward in certain aspects of this work.

* This work was supported in part by a research contract, with the Atomic Energy Commission, AT (30-1)-872; by a pre-doctoral fellowship, MF-7405, of the National Institute of Mental Health, Public Health Service held by the senior author; and by Institutional Grant 47F from the American Cancer Society.

[1] N. H. Giles, C. W. H. Partridge, and N. J. Nelson, these PROCEEDINGS, **43**, 305, 1957; and *Proc. Japan. Genetics Symposia*, Supplement volume of *Cytologia*, p. 543, 1956.

[2] J. R. S. Fincham and J. A. Pateman, *Nature*, **179**, 741, 1957.

[3] A. M. Lacy and D. M. Bonner, *Proc. X Intern. Congr. Genetics*, **2**, 157, 1958 (abstr.).

[4] M. E. Case and N. H. Giles, these PROCEEDINGS, **44**, 378, 1958.

[5] D. G. Catcheside and A. Overton, *Cold Spring Harbor Symposia Quant. Biol.*, **23** (in press).

[6] Abbreviations used are SAICAR = 5-amino-4-imidazole(N-succinylocarboxamide) ribotide; and AICAR = 5-amino-4-imidazolecarboxamide ribotide.

[7] N. H. Giles, *Proc. X Intern. Cong. Genetics*, **1** (in press), 1958.

[8] D. O. Woodward, *Proc. X Intern. Cong. Genetics*, **2**, 321, 1958 (abstr.).

[9] V. W. Woodward, J. R. de Zeeuw, and A. M. Srb, these PROCEEDINGS, **40**, 192, 1954.

[10] N. J. Nelson, unpublished Ph.D. thesis, Yale University, 1957.

[11] F. J. de Serres, *Genetics*, **41**, 668, 1956.

[12] F. M. Richards, these PROCEEDINGS, **44**, 162, 1958.

[13] H. A. Itano, *Abstr. Papers 134th Meeting Am. Chem. Soc.*, 1958, (abstr.).

[14] A. G. Bearn and E. C. Franklin, *Science*, **128**, 596, 1958.

[15] M. E. Case, and N. H. Giles, *Cold Spring Harbor Symposia Quant. Biol.*, **23**, (in press), and unpublished data.

---

*COHOMOLOGY THEORY OF VARIETIES OVER RINGS**

BY WEI-LIANG CHOW AND JUN-ICHI IGUSA

DEPARTMENT OF MATHEMATICS, JOHNS HOPKINS UNIVERSITY

Communicated by Oscar Zariski, October 13, 1958

1. *Introduction.*—Some recent developments in algebraic geometry, e. g., the theory of deformations of complex analytic structures by Kodaira and Spencer, investigations on the principles of degeneration and of connectedness by Chow and on elliptic modular functions by Igusa, suggest the importance of the notion of varieties considered not merely over fields but more generally over rings. This

type of varieties gradually took shape in some works of Zariski, and a tentative formulation was given by Nagata rather recently.[1] In this short note, we shall outline a cohomology theory of such varieties and derive, among others, the *principle of upper semicontinuity* as a corollary of a Künneth relation in our cohomology theory.

2. *Models and Cohomology.*—We fix a field Σ and consider a subring A which is Noetherian and which has Σ as its field of fractions. The totality of rings of fractions constructed from A with respect to prime ideals is called a *chunk*. A local ring of Σ is called a *spot*. Two chunks U_1 and U_2 defined, respectively, by A_1 and A_2 may be called coherent if the compositum A_1A_2 is a finite ring extension of A_1 and A_2, hence Noetherian, and if the chunk defined by A_1A_2 is precisely the intersection of U_1 and U_2. A non-empty set X of spots is called a *model* of Σ, or simply a model, if it can be expressed as a union of a finite number of coherent chunks. If f is an element of Σ, the set of spots of X containing f is denoted by D_f. The totality of D_f generates a topology in X, which is called the Zariski topology of X. An open chunk contained in X may be called a *neighborhood*. Observe that the finite number of chunks by which X was defined are neighborhoods. Also, every open set is a union of neighborhoods, and every increasing sequence of open sets is necessarily finite.

Since a model X is a topological space, we can consider sheaves on X. In fact, the algebraic sheaf theory as developed by Serre[2] can be taken over verbatim to the present case. We get the sheaf of local rings, algebraic sheaves, coherent sheaves, locally free sheaves, etc. A crucial lemma, known as Hilbert's "Nullstellensatz," is in our case a triviality, because we are not restricting "points" to those which correspond to maximal ideals. If F is a sheaf on X, the *cohomology group* of X with coefficients in F, in notation $H(X,F)$, is obtained by Čech method: If U is a finite open covering of X, the module of q-cochains of U with coefficients in F is denoted by $C^q(U,F)$. The graded module $C(U,F) = \Sigma\, C^q(U,F)$ is a complex with a coboundary operator δ. The corresponding cohomology group is denoted by $H(U,F)$. One then passes to the direct limit of the groups $H(U,F)$ and one obtains the cohomology group $H(X,F)$. The following theorems are fundamental in our cohomology theory.

THEOREM 1. *Let F be a coherent sheaf on a model X which can be covered by one neighborhood. Then we have $H^q(X,F) = 0$ for $q > 0$.*

THEOREM 2. *Let F be a coherent sheaf on a model X and let U be a finite covering of X by coherent neighborhoods. Then the homomorphism $H(U,F) \to H(X,F)$ is an isomorphism.*

As it follows from Theorem 1 and Theorem 2, if $0 \to F' \to F \to F'' \to 0$ is an exact sequence of sheaves on X such that F' is coherent, the corresponding cohomology sequence

$$\to H^q(X,F') \to H^q(X,F) \to H^q(X,F'') \xrightarrow{\delta^*} H^{q+1}(X,F') \to$$

is exact. The third theorem is more delicate, and we must make some restrictions. Suppose that R is a fixed Noetherian domain such that Σ is a finitely generated extension of its field of fractions, say K. Then a finite ring extension of R with Σ as its field of fractions is Noetherian; hence it defines a chunk. If a model X is covered by this type of neighborhoods, we may call it a *geometric model* over R.

The cohomology groups of such models with coefficients in algebraic sheaves are R-modules. There is a classical way of getting geometric models: Pick a finite number of elements $x_0 = 1, x_1, \ldots, x_r$ of Σ which are different from zero and which generate Σ over K. With respect to such choice of generators, we get finite ring extensions $A_i = R[x_0/x_i, \ldots, x_r/x_i]$ of R for $i = 0, 1, \ldots, r$. The corresponding $r + 1$ chunks are coherent; hence their union X is a model. In fact, X is a geometric model over R, and it is called a *projective model* over R. We can now state our third theorem:

THEOREM 3. *If R is a regular local ring and if X is a projective model over R, the cohomology groups of X with coefficients in coherent sheaves are finite R-modules.*

Again, the proof is almost the same as in Serre. It is necessary, however, to have the Hilbert theorem on "chains of syzygies" in the following generalized form:[3]

LEMMA. *Let A be a ring of polynomials in $r + 1$ letters with coefficients in a regular local ring. Then every graded finite A-module admits a finite free resolution.*

We can apply Theorem 3 to the investigation of cohomology of "fiber systems" over non-singular models. In the next section, we shall discuss the simplest non-trivial case, i. e., the case where the base space is defined by a regular local ring of one dimension.

3. *A Künneth Relation.*—Keeping the notation of the previous section, assume that R is a discrete valuation ring with πR as its ideal of non-units and with k as the corresponding residue field. Let X be a projective model over R. The spots of X containing K or, more suggestively, not on the hyperplane $\pi = 0$, form an open subset Y of X. Observe that Y is a projective model over K. On the other hand, we shall *assume* that the tensor product $Y' = k \otimes_R X$ defined in an obvious way is a model over k. Let F be a coherent sheaf on X. Then, the restriction F_Y of F to Y is a coherent sheaf on Y and the tensor product $F' = k \otimes_R F$ is a coherent sheaf on X, which can be considered also as a coherent sheaf on Y'.

Now, consider the free resolution

$$0 \to R \xrightarrow{\pi} R \to k \to 0$$

of k and tensorize this exact sequence by a coherent sheaf F on X. We shall *assume* that the tensorized sequence is exact. Let U be a finite covering of X by coherent neighborhoods and consider the cochain complex $C(U,F)$. Then the Künneth relation[4] for the above complex over k and $C(U,F)$ takes the following form:

$$0 \to k \otimes_R H(X,F) \to H(Y',F') \to Tor_1^R(k,H(X,F)) \to 0.$$

Now, in general, if for an R-module M we denote by ${}_\pi M$ its submodule of elements m satisfying $\pi m = 0$, the "Tor" can be identified with ${}_\pi H(X,F)$. For the convenience of the reader, we shall give a proof of the above relation in a few lines: By assumption, the sequence $0 \to F \xrightarrow{\pi} F \to F' \to 0$ is exact; hence we get the corresponding cohomology sequence,

$$\to H^q(X,F) \xrightarrow{\pi^*} H^q(X,F) \to H^q(Y',F') \xrightarrow{\delta^*} H^{q+1}(X,F) \to.$$

Also, if we tensorize the exact sequence $0 \to R \xrightarrow{\pi} R \to k \to 0$ by $H(X,F)$, we get the following exact sequence:

$$0 \to {}_\pi H(X,F) \to H(X,F) \xrightarrow{\pi^*} H(X,F) \to k \otimes_R H(X,F) \to 0.$$

The combination of these two exact sequences will give the Künneth relation.

On the other hand, the restriction of F to Y gives rise to an R-homomorphism $H(X,F) \to H(Y,F_Y)$. We shall show that the image generates $H(Y,F_Y)$ over K and the kernel is the torsion submodule of $H(X,F)$: As before, take a finite covering U of X by coherent neighborhoods. Then the restriction U_Y of U to Y is a finite covering of Y by coherent neighborhoods. Moreover, if f is an element of $C(U_Y,F_Y)$, there exists a positive integer m such that $\pi^m f$ is the image of an element, say g, of $C(U,F)$. If f is a cocycle, there exists a positive integer n such that $\pi^n g$ become a cocycle. The first part is thereby proved. The proof of the second part is similar. Therefore, we can identify the tensor product $K \otimes_R H(X,F)$ with $H(Y,F_Y)$. In particular, we have

$$\dim k \otimes_R H^q(X,F) = \dim H^q(Y,F_Y) + \dim {}_\pi H^q(X,F).$$

If we combine this with the previous relation, we finally get the following *fundamental relation:*

$$\dim H^q(Y',F') - \dim H^q(Y,F_Y) = \dim {}_\pi H^q(X,F) + \dim {}_\pi H^{q+1}(X,F).$$

Observe that $H^0(X,F)$ is R-free. At any rate, if χ denotes the Euler-Poincaré characteristic, we have $\chi(Y',F') = \chi(Y,F_Y)$. We shall show in the next section that our fundamental relation implies the principle of upper semicontinuity.

4. *Principle of Upper Semicontinuity.*—We shall assume that $\Sigma = K(x)$ is a regular extension of K. Then $(x) = (x_0, x_1, \ldots, x_r)$ can be considered as a generic point of a projective variety V over K. Let V' be its specialization at the center of R. We shall assume that V' is a variety. The generators $x_0, x_1, \ldots, x_r$ of Σ over K define a projective model over R. We take its derived normal model in Σ and call it X. We note that X is also a projective model over R.[5] Moreover, if we assume that V and V' are locally normal,[6] we can identify the open subset Y of X of spots not on the hyperplane $\pi = 0$ with the totality of local rings of V over K and $Y' = k \otimes_R X$ with the totality of local rings of V' over k. In particular Y' is a projective model over k. Let E be a vector bundle on V defined over K such that its specialization E' at the center of R is a vector bundle on V'. Then we get sheaves of germs of sections of E and E'. If we denote these sheaves by $S(E)$ and $S(E')$, they are locally free sheaves on V and V'. Also E defines a locally free sheaf F on X in an obvious way. As before, we shall denote the restriction of F to Y by F_Y and $k \otimes_R F$ by F'. Then the sequence $0 \to F \xrightarrow{\pi} F \to F' \to 0$ is certainly exact. Moreover, if Ω and Ω' are the universal domains over K and k, by a result of Weil[7] we can identify $H(V,S(E))$ with $\Omega \otimes_K H(Y,F_Y)$ and $H(V',S(E'))$ with $\Omega' \otimes_k H(Y',F')$. In this way, we get the following theorem:

THEOREM 4. *Let V be a projective variety and let V' be its equal or unequal-characteristic specialization. We shall assume that V' is a normal variety. Let E be a vecor bundle on V which is specialized to a vector bundle E' on V' over the above specialization. Let $S(E)$ and $S(E')$ be the sheaves of germs of sections of E and E'. Then $\dim H^q(V',S(E'))$ is at least equal to $\dim H^q(V,S(E))$ and we have $\chi(V',S(E')) = \chi(V,S(E))$. In particular V and V' have the same arithmetic genus.*

We note that the last statement was already proved by a different method some

years ago.[8] Also, we note that the "pathology" of unequal-characteristic specializations[9] is explained quantitatively by the fundamental relation in the previous section.

* This work was partially supported by the National Science Foundation.

[1] M. Nagata, "A General Theory of Algebraic Geometry over Dedekind Domains. I," *Am. J. Math.*, **78**, 78–116, 1956.

[2] J.-P. Serre, "Faisceaux algébriques cohérents," *Ann. Math.*, **61**, 197–278, 1955.

[3] This lemma is implicitly proved in Cartan-Eilenberg, *Homological Algebra* (Princeton, N. J.: Princeton University Press, 1956), pp. 154–156.

[4] *Ibid.*, pp. 112–116.

[5] Cf. Nagata, *op. cit.*, p. 104.

[6] Actually, the local normality of V' implies the local normality of V. Cf. J. Igusa, "Arithmetic Genera of Normal Varieties in an Algebraic Family," these PROCEEDINGS, **41**, 34–37, 1955.

[7] A. Weil, *Foundations of Algebraic Geometry* ("Am. Math. Soc. Colloquium Publs.," No. 29 [1946]), p. 239.

[8] Cf. Igusa, *op. cit.*

[9] J. Igusa, "On Some Problems in Abstract Algebraic Geometry," these PROCEEDINGS, **41**, 964–967, 1955. The footnote 12 is incorrect; the surface V_2 is indeed a specialization of V_0. This fact was remarked orally to the second-named author by Mr. Hironaka.

ON THE FOUNDATIONS OF THE THEORY OF DISTRIBUTIONS

BY I. S. GÁL

CORNELL UNIVERSITY AND YALE UNIVERSITY

Communicated by Einar Hille, October 13, 1958

This note is a summary of results obtained by considering Schwartz distributions as the elements of the completion of a suitable uniform structure $\mathfrak{U}$ for the linear space $\mathfrak{D}$ of those real-valued functions on a finite dimensional Euclidean space S which have compact supports and possess mixed derivatives of all orders on S.

DEFINITION. *A distribution structure for an inner product space X is a uniform structure associated with a family of sets $U \subseteq X$ having the property that for every U, $V \in \mathfrak{U}$ there is a $W \in \mathfrak{U}$ satisfying $U \cup V \subseteq W$. The uniform structure associated with $\mathfrak{U}$ is determined by the semi-norms $\|x\|_U$ where $\|x\|_U = \sup \|x\|_u$ and $\|x\|_u = |(x, u)|$.*

A simple non-trivial distribution structure can be introduced on every inner product space by choosing $\mathfrak{U}$ to be the family of all finite subsets of X. The corresponding separated structure is called the uniform structure of weak distributions. The uniform structure of Schwartz distributions on a finite dimensional Euclidean space S is defined as follows: The inner product space X consists of all real-valued functions x on S which have compact support and possess partial derivatives of all orders on S. By $[x]$ we shall denote the smallest closed convex support of x and if $U \subseteq X$, then $[U]$ will be the smallest closed convex set in S which contains every $[u]$ $(u \in U)$. We use the notations $\|x\| = \sup |x(s)|$ and $\|U\| = \sup \|u\|$, where $u \in U$ and $U \subset X$. The inner product is $(x, y) = \int xy d\mu$, where μ denotes the n-dimensional Lebesgue measure. Let $\mathfrak{a}$ denote the semigroup of all mixed derivatives $A : X \to X$. Then $U \in \mathfrak{U}$ if and only if the elements of U

have a common compact support and if $\|AU\| < +\infty$ for every $A \in \mathfrak{A}$. The uniform structure associated with the family $\mathfrak{U}$ is called the structure of Schwartz distributions on S. Let Y be the linear space of those real-valued functions y on S which have mixed derivatives of all orders on S and let Z be the linear space of Lebesgue measurable functions z on S which can be integrated on sets of finite measure. One proves that X and Y are dense in Z relative to the uniform topology associated with the structure of Schwartz distributions. Hence X, Y, and Z have identical completion $\bar{X} = \bar{Y} = \bar{Z}$ and so $Z \subseteq \bar{X}$. Other distribution structures can be introduced on X by using sets $U \subseteq Y$ instead of subsets of X. These and further generalizations, such as vector distributions, will not be discussed here.

The set X with distribution structure $\mathfrak{U}$ has a unique completion $\bar{X}$ with respect to $\mathfrak{U}$. The elements of $\bar{X}$ are the distributions with respect to $\mathfrak{U}$, and the completed structure is denoted by $\bar{\mathfrak{U}}$. If $\mathfrak{U}$ is the uniform structure of Schwartz distributions, then it can be proved that $\bar{X}$ is uniformly isomorphic to the topological vector space $\mathfrak{D}'$ introduced by Schwartz. The partial differential operators $A:X \to X$ can be extended in a unique way to operators $A:\bar{X} \to \bar{X}$. For every $A \in \mathfrak{A}$ is uniformly continuous on X with respect to the uniform structure $\mathfrak{U}$ and X is dense in $\bar{X}$. The continuity of A can be proved as follows: An operator $P:X \to X$ is called a *local operator* with respect to the uniform structure $\mathfrak{U}$ of Schwartz distributions if $[PU]$ is compact for every $U \in \mathfrak{U}$. It is called *bounded* with respect to $\mathfrak{U}$ if $\|APU\| < +\infty$ for every $A \in \mathfrak{A}$ and $U \in \mathfrak{U}$.

LEMMA 1. *If $A:X \to X$ has an adjoint $A^*:X \to X$ which is bounded and local with respect to the uniform structure of Schwartz distributions $\mathfrak{U}$ then A is uniformly continuous with respect to $\mathfrak{U}$.*

A number of linear operators $A:X \to X$ satisfy the requirements of the lemma. Since the underlying space S is a linear space, we can speak about the translation $T^s:X \to X$ by an element $s \in S$ and about the similarity transformation $S_\lambda:X \to X$, where $\lambda \neq 0$. These are bounded and local operators, and we have $(T^s)^* = T^{-s}$ and $(S_\lambda)^* = \lambda^{-1}S_\lambda - I$. The rules $T^sT^t = T^{s+t}$ and $S_\lambda S_\mu = S_{\lambda\mu}$ hold for the extended operators. If $a \in Y$, then a linear operator A is defined on X by the formula $Ax = ax$. Since A is self-adjoint, local, and bounded, it can be extended to a continuous linear operator $A:\bar{X} \to \bar{X}$. The product of a distribution $x \in \bar{X}$ by the function $a \in Y$ is defined as $Ax = ax$. The rules $(ab)x = a(bx)$ and $(ax)_i = a_ix + ax_i$ follow from the continuity of the product and partial differentiation operators. The tensor product of distributions can be defined by proving the continuity of the linear operator mapping $X^m \times X^n$ into X^{m+n} according to the rule $(x^m \otimes x^n)(s, t) = x^m(s)x^n(t)$.

For fixed $x \in X$, the inner product (x, ξ) is a continuous functional on X with respect to the structure $\mathfrak{U}$ of Schwartz or weak distributions. Hence it can be extended to a bilinear functional (x, ξ) over $X \times \bar{X}$, which is continuous for every fixed $x \in X$ in its distribution variable $\xi \in \bar{X}$. For a fixed value of ξ the formula $\xi(x) = (\xi, x)$ defines a (discontinuous) linear functional on X which is zero if and only if $\xi = \theta$. Hence distributions can be represented as linear functionals on X. Schwartz's definition of a distribution is given in terms of these representations. Every linear functional is a weak distribution. A simple direct proof can be given for the following result of Schwartz:

A linear functional λ *is a Schwartz distribution if and only if* $\|\lambda\|_U$ *is finite for every* $U \in \mathfrak{U}$.

Now let S be the real line and let $\mathfrak{U}$ be the uniform structure of Schwartz distributions. A formal interpretation of a property of the Dirac delta which is used in physics is the formula

$$2a\delta(s^2 - a^2) = \delta(s - a) + \delta(s + a),$$

where $a > 0$. This formula is meaningful in the theory of distributions: We consider the linear map $A:X \to X$ which is given by $(Ax)(s) = x(s^2 - a^2)$. Let L denote the linear space of those $x \in X$ which vanish outside of $[-a^2/4, +a^2/4]$. Then δ belongs to the closure of L and the restriction of A to L is uniformly continuous with respect to $\mathfrak{U}$. Hence the image of δ under A is meaningful. The formula in question means $2aA\delta = (T^{-a} + T^a)\delta$. Its proof is based on

LEMMA 2. *If* ξ *is a Schwartz distribution of compact support on the real line then*

$$\lim_{|\lambda| \to +\infty} (\lambda S_\lambda \xi) = (\xi, 1)\delta.$$

Here convergence is understood with respect to the topology of Schwartz distributions and the index set $\Lambda = S - \{0\}$ is ordered by "$\lambda_1 \leq \lambda_2$ *if and only if* $|\lambda_1| \leq |\lambda_2|$." Since ξ has compact support, the inner product (ξ, y) is meaningful for every $y \in Y$. Using the same method, we can prove that $2na^{2n-1}A\delta = (T^{-a} + T^a)\delta$, where A is given on X by $(Ax)(s) = x(s^{2n} - a^{2n})$ and $a > 0$. For odd exponents we have $(2n + 1)a^{2n}A\delta = T^a\delta$. If S is the Euclidean plane and st denotes complex multiplication, then we have similar expressions for the delta function on S.

The present method can be successfully applied to the problem of inversion of continuous linear operators. Let S be a finite dimensional Euclidean space and let $A:X \to X$ be a continuous linear operator with adjoint $A^*:X \to X$ such that $[u:A^*u \in W] \in \mathfrak{U}$ for every $W \in \mathfrak{U}$. The range and the null-space of $A^*:X \to X$ will be denoted by R^* and Z^*, respectively. The symbols R and Z will be used to denote the range and the null-space of the extended operator $A:\bar{X} \to \bar{X}$. If the linear space $E \subseteq X$ is maximal with respect to the property $E \cap R^* = \{\theta\}$, then for every $x \in X$ there is a unique $e \in E$ such that $x = e + r^*$ for some $r^* \in R^*$. We define an idempotent linear map $P_E:X \to E$ by setting $P_E = e$.

LEMMA 3. *Let* E *be such that* P_E *is bounded and local. Then for every* $r \in R$ *there is a unique distribution* z *such that* $x - z$ *is orthogonal to* E *and* $z \in Z$.

This lemma is proved by constructing a linear functional on X which represents the distribution z. A linear operator $A^{-1}:R \to \bar{X}$ can be defined now such that $A^{-1}r$ is orthogonal to E and $AA^{-1}r = r$ for every $r \in R$. Namely, we define $A^{-1}r = x - z$, where $r = Ax$ and z is the distribution introduced in the foregoing lemma.

LEMMA 4. *The linear map* $A^{-1}:R \to \bar{X}$ *is continuous.*

This follows from the existence of an adjoint operator A^* with the additional property mentioned above. The map A^{-1} being uniformly continuous with respect to the uniform structure of Schwartz distributions, it has a unique continuous extension $A^{-1}:\bar{R} \to \bar{X}$. If the only solution of $A^*u = \theta$ in X is $u = \theta$ then $R = \bar{R} = \bar{X}$.

THEOREM 1. *Let* $A:X \to X$ *be a continuous linear operator with adjoint* $A^*:X \to X$ *such that* $[u:A^*u \in W] \in \mathfrak{U}$ *for every* $W \in \mathfrak{U}$, *and let* E *be a linear subspace of* X *such*

that $X = E \oplus R^*$ and the map $P_E: X \to E$ is bounded and local. Then there exists a unique continuous linear operator $A^{-1}: \bar{R} \to \bar{X}$ whose range is orthogonal to E and satisfies $AA^{-1} = I$ on $\bar{R}$.

The simplest non-trivial application of this result occurs in the problem of continuous antidifferentiation of distributions over the real line S. One obtains the following:

THEOREM 2. *Given any function $e \in X$ satisfying $(e, 1) \neq 0$, there is a unique continuous linear operator $D^{-1}: \bar{X} \to \bar{X}$ such that $DD^{-1} = I$ and $D^{-1}x$ is orthogonal to e for every $x \in \bar{X}$.*

Let $a_0, \ldots, a_{n-1}$ be arbitrarily often differentiable functions on the real line and let A be the differential operator $A = \sum a_k D^k + D^n$. Given a fundamental system $y_1, \ldots, y_n$ of solutions of $Ay = \theta$, let $W = W(y_1, \ldots, y_n)$ be the Wronskian and let W_k $(k = 1, \ldots, n)$ be the cofactor of $y_k^{(n-1)}$ in W. We can prove that if D_1^{-1}, $\ldots$, D_n^{-1} are continuous antidifferentiation operators, then the linear operator

$$A^{-1} = y_1 D_1^{-1} W_1 W^{-1} + \ldots + y_n D_n^{-1} W_n W^{-1}$$

maps $\bar{X}$ continuously into itself and is such that $AA^{-1} = I$. A second way of selecting continuous linear operators A^{-1} is by applying Theorem 1. We obtain:

THEOREM 3. *Given any differential operator*

$$A = a_0 I + a_1 D + \ldots + a_{n-1} D^{n-1} + D^n$$

with $a_0, \ldots, a_{n-1}$ in Y and any linear space E satisfying $X = E \oplus R^*$, there is a unique continuous linear operator $A^{-1}: \bar{X} \to \bar{X}$ such that the range of A^{-1} is orthogonal to E and $AA^{-1} = I$.

There is a large class of linear spaces E satisfying the requirements, for $X = E \oplus R^*$ if and only if E is n-dimensional and for large values of s it coincides with Z^*.

The difference operator $\Delta: \bar{X} \to \bar{X}$ is first defined on X by the formula $(\Delta x)(s) = x(s + 1) - x(s)$ and then extended by continuity to the whole of $\bar{X}$. Using Theorem 1, we can prove the existence of continuous linear operations $\Delta^{-1}: \bar{X} \to \bar{X}$ such that $\Delta\Delta^{-1} = I$. More precisely, if e is a non-negative function of X which does not vanish on some interval of length greater than 1, then there exists a unique operator Δ^{-1} such that $\Delta\Delta^{-1} = I$ and $\Delta^{-1}x$ is orthogonal to pe for every $x \in \bar{X}$ and every periodic $p \in Y$.

The operator Δ is a special instance of the operators

$$A = a_0 I + a_1 T + \ldots + a_{n-1} T^{n-1} + T^n,$$

where T denotes translation by 1. We suppose that $a_0, \ldots, a_{n-1}$ are analytic functions which are real and regular on S, and we suppose also that a_0 has no roots on S. Then the homogeneous equation $Ay = \theta$ has a fundamental system of analytic solutions $y_1, \ldots, y_n$ which are real and regular on S. It is known that the determinant $W = W(y_1, \ldots, y_n) = |T^i y_j|$ $(i, j = 1, \ldots, n)$ never vanishes on S. Let W_k $(k = 1, \ldots, n)$ denote the cofactor which corresponds to the element $T^n y_k$ and let Δ_k^{-1} $(k = 1, \ldots, n)$ be continuous inverses of the difference operator Δ. Then we can prove that the linear operator

$$A^{-1} = y_1 \Delta_1^{-1} W_1 W^{-1} + \ldots + y_n \Delta_n^{-1} W_n W^{-1}$$

is a continuous inverse of A, that is, $AA^{-1} = I$ on $\bar{X}$.

THEOREM 4. *Let a be an arbitrarily often differentiable function on the real line having countably many isolated zeros $s_1, s_2, \ldots$ of finite orders $m_1, m_2, \ldots$, respectively. Denote by A the multiplication operator mapping x into $Ax = ax$ and let E be any linear space satisfying $X = E \oplus R^*$. Then there is a unique continuous linear operator $A^{-1}:\bar{X} \to \bar{X}$ whose range is orthogonal to E and is such that $AA^{-1} = I$.*

The boundedness and local property of P_E can be proved by using the fact that every E satisfying $E \cap R^* = \{\theta\}$ is locally finite dimensional. Precisely, $X = E \oplus R^*$ if and only if for every root s_k there exist elements $\epsilon_\lambda \in E$ ($\lambda = 0, \ldots, m_k - 1$) with the property that $\epsilon_\lambda^{(\mu)}(s_k) = \delta_{\lambda\mu}$ for $\lambda, \mu = 0, \ldots, m_k - 1$, where $\delta_{\lambda\mu}$ is the Kronecker delta and at each s_n ($n \neq k$) the functions ϵ_λ vanish with order m_n. A natural division operator $(A^{-1})^*:R^* \to X$ is defined on R^*, which maps $r^* \in R^*$ into $r^*/a \in X$. Although the operator $(A^{-1})^*$ is not continuous, it can be extended in many ways to a linear operator $(A^{-1})^*:X \to X$. Namely, each A^{-1} has an adjoint $(A^{-1})^*:X \to X$ whose restriction to R^* is the natural division operator. The null-space of the adjoint operator associated with E is the linear space E.

The crucial point in the proof is the verification of the condition "$[u:au \in W]$ *belongs to* $\mathfrak{U}$ *for every* W *in* $\mathfrak{U}$." The only non-trivial tool needed in the proof of this proposition is the generalized mean-value theorem of the differential calculus. The same proposition leads to the solution of the division problem for special types of divisors in the case of several variables. This includes division by linear functions and by quadratic functions of the form $a(s_1, \ldots, s_n) = \sum a_k s_k^2$, where $a_k \geq 0$ ($k = 1, \ldots, n$). Earlier Schwartz proved that division of individual distributions by "regular" functions is possible.[1] The principle of localization and a change in variables show that division by regular functions can be treated globally:

THEOREM 5. *Let a be a regular function on a finite dimensional Euclidean space S and let $A:\bar{X} \to \bar{X}$ be the operator corresponding to multiplication by a. Then there exist continuous linear operators $A^{-1}:\bar{X} \to \bar{X}$ such that $AA^{-1} = I$.*

A satisfactory solution of the division problem in general would depend on the proof of the proposition "$[u:au \in W]$ *belongs to* $\mathfrak{U}$ *for every* W *in* $\mathfrak{U}$."

[1] L. Schwartz, *Théorie des distributions* ("Actualités scientifiques et industrielles," No. 1245 [Paris: Hermann, 1957]), **1**, 126–28.

Note added in proof: I was recently informed that the problem of division of fixed distributions was solved by both L. Hörmander and S. Lojasiewicz working independently.

ON THE SPECTRAL THEORY OF SINGULAR INTEGRAL OPERATORS

BY WALTER KOPPELMAN*

DEPARTMENT OF MATHEMATICS, YALE UNIVERSITY

Communicated by Einar Hille, September 29, 1958

The purpose of this note is to present some results on the spectral theory of singular integral operators of the form

$$Lx(\lambda) = f(\lambda)x(\lambda) + \frac{1}{\pi i}\int_a^b \frac{\sqrt{k(\lambda)k(\mu)}}{\mu - \lambda}\, x(\mu)d\mu$$

acting on functions $x(\lambda)$ belonging to the class $L^2(a, b)$. (The integral which

appears here is to be interpreted as a Cauchy principal value.) We shall assume that $f(\lambda)$ and $k(\lambda)$ are real, continuously differentiable functions on the interval $a \leq \lambda \leq b$, such that the functions $f'(\lambda) \pm k'(\lambda)$ have only isolated zeros and where $k(\lambda) > 0$ almost everywhere on the interval $a \leq \lambda \leq b$.

From the theory of singular integral equations[1] and some results on integrals of Cauchy type,[2] one can prove

THEOREM 1. *The limit points of the spectrum of the operator L lie in the closed interval $[A, B]$, where $A = \min\{f(\lambda) - k(\lambda)\}$, $B = \max\{f(\lambda) + k(\lambda)\}$.*

Next we introduce the functions

$$E(l, z) = \exp\left\{\frac{1}{2\pi i}\int_a^b \log\frac{f(u) - l - k(u)}{f(u) - l + k(u)}\,\frac{du}{u - z}\right\},$$

$$H(l, \lambda) = E(l, \lambda + i0) - E(l, \lambda - i0),$$

$$F(\xi, z) = E(\xi + i0, z) - E(\xi - i0, z),$$

and

$$A(\xi) = -\frac{1}{2\pi i}\lim_{\substack{\eta \to 0 \\ \eta > 0}} \int_a^b \{H(\xi + i\eta, \lambda) - H(\xi - i\eta, \lambda)\}d\lambda.$$

Here ξ and λ are real. Then one can prove

LEMMA 1. *$A(\xi) > 0$ almost everywhere on the interval $[A, B]$.*

and

THEOREM 2. *The transformations*

$$\hat{R}h(\lambda) = -\frac{1}{2\pi i}\lim_{\substack{\eta \to 0 \\ \eta > 0}} \int_a^b \frac{\overline{H(\xi + i\eta, \lambda) - H(\xi - i\eta, \lambda)}}{\sqrt{2k(\lambda)A(\xi)}}\, h(\lambda)d\lambda$$

and

$$\hat{S}g(\xi) = \frac{1}{2\pi i}\lim_{\substack{\eta \to 0 \\ \eta > 0}} \int_A^B \frac{F(\xi, \lambda + i\eta) - F(\xi, \lambda - i\eta)}{\sqrt{2k(\lambda)A(\xi)}}\, g(\xi)d\xi$$

generate isometries R and S between the subspace $\bar{L}_\lambda$ of $L^2(a, b)$, which is the closure of the linear manifold L_λ generated by the elements $\left\{\sqrt{2k(\lambda)}, \frac{H(\omega, \lambda)}{\sqrt{2k(\lambda)}}\right\}$ where ω ranges over all complex numbers in the exterior of the interval $[A, B]$ and the space $L^2(A, B)$. S is the inverse of R. The space L_λ is invariant under the operation L. Furthermore, if $h(\lambda)$ belongs to $\bar{L}_\lambda$ and if $g(\xi) = Rh(\lambda)$, then $\xi g(\xi) = RLh(\lambda)$.[3]

From Theorems 1 and 2 we obtain

COROLLARY. *The set of limit points of the spectrum of the operator L is the entire interval $[A, B]$ and if $k(\lambda) \neq 0$, $a \leq \lambda \leq b$, then the spectrum of L consists of precisely all the points of the interval above.*

Now, one is in the position to prove

THEOREM 3. *Consider the function $G(\lambda; \xi) = \{f(\lambda) - \xi - k(\lambda)\}/\{f(\lambda) - \xi + k(\lambda)\}$, $a \leq \lambda \leq b$. Suppose that when $f(a) - k(a) \leq \xi \leq f(a) + k(a)$, the function $G(\lambda; \xi)$ changes sign at most once in the interval $a \leq \lambda \leq b$, and that for all other values of ξ,*

the function changes signs at most twice. Then the operator L has a simple continuous spectrum from $A = min\{f(\lambda) - k(\lambda)\}$ to $B = max\{f(\lambda) + k(\lambda)\}$ and the transformations R and S of Theorem 2 furnish a complete spectral representation for L.

$F(\xi, z)$ satisfies an identity of the form

$$\frac{(z_2 - z_1)P(\xi, z_1, z_2)}{Q(\xi, z_1)Q(\xi, z_2)} F(\xi, z_1)\,\overline{F(\xi, \bar{z}_2)} = \{E(\xi + i0, z_1)\,\overline{E(\xi - i0, \bar{z}_2)} - E(\xi - i0, z_1)\,\overline{E(\xi + i0, \bar{z}_2)}\},\ A < \xi < B,$$

where for each fixed ξ, $P(\xi, z_1, z_2)$ is a polynomial in z_1 and z_2 with real coefficients and $Q(\xi, z)$ is a polynomial in z with real coefficients. We now make the following hypothesis:

HYPOTHESIS A.

$$\frac{P(\xi, z_1, z_2)}{Q(\xi, z_1)Q(\xi, z_2)} = \frac{1}{A(\xi)} + \sum_{j=1}^{N} \frac{C_j(\xi, z_1)C_j(\xi, z_2)}{Q(\xi, z_1)Q(\xi, z_2)},$$

where the $C_j(\xi, z)$ are polynomials in z, with real coefficients.

One can now prove

LEMMA 2. *Suppose that when $f(a) - k(a) \leq \xi \leq f(a) + k(a)$, the function $G(\lambda; \xi)$ of Theorem 3 changes signs at most three times in the interval $a \leq \lambda \leq b$, and that for all other values of ξ, the function changes signs at most four times. Then hypothesis A is satisfied and*

$$\frac{P(\xi, z_1, z_2)}{Q(\xi, z_1)Q(\xi, z_2)} = \frac{1}{A(\xi)} + \frac{\sqrt{B(\xi)/A(\xi)}}{Q(\xi, z_1)} \frac{\sqrt{B(\xi)/A(\xi)}}{Q(\xi, z_2)},$$

where $B(\xi) \geq 0$.

THEOREM 4. *If the function $G(\lambda; \xi)$ satisfies the conditions stated in Lemma 2, then there exists an isometry $\mathfrak{R}$ of $L^2(a, b)$ onto the direct sum $\mathfrak{K}$ of the two Hilbert spaces $L^2(A, B)$ and $L^2(\sum)$, where $\sum = \{\xi | B(\xi) \pm 0, A < \xi < B\}$.* $\mathfrak{R}$ "coincides" with the transformation R of Theorem 2 on the subspace $\bar{L}_\lambda$ of $L^2(a, b)$. Furthermore, $\mathfrak{R}$ furnishes a spectral representation of the operator L. Thus the spectrum of L has multiplicity ≤ 2.

More general self-adjoint operators of the form

$$Mx(\lambda) = f(\lambda)x(\lambda) + \frac{1}{\pi i}\int_a^b \frac{g(\lambda, \mu)}{\mu - \lambda} x(\mu)d\mu$$

can be shown differ from a direct sum of operators of the form L and multiplication operators by completely continuous operators. Thus we have a method for obtaining the essential spectrum of operators of the form M.

* This research was performed under the auspices of the Atomic Energy Commission, while the author was a Visiting Mathematician at Brookhaven National Laboratory.

[1] N. S. Muskhelishvili, *Singular Integral Equations* (Groningen: Noordhoff, 1953).

[2] E. C. Titchmarch, *Introduction to the Theory of Fourier Integrals* (Oxford, 1937).

[3] W. Koppelman and J. D. Pincus, "Perturbation of Continuous Spectra and Singular Integral Operators," *Comm. on Pure and Appl. Math.* (to appear), contains a special case of this result.

*INDIVIDUAL DIFFERENCES IN SUSCEPTIBILITY TO HYPNOSIS**

By Ernest R. Hilgard, André M. Weitzenhoffer, and Philip Gough

DEPARTMENT OF PSYCHOLOGY, STANFORD UNIVERSITY

Communicated September 27, 1958

While all investigators report wide individual differences in susceptibility to hypnosis, the form of the distribution is a matter of some uncertainty. Some years ago Hull[1] concluded that "susceptibility to hypnotic suggestion is fairly evenly distributed over the range from no susceptibility whatever to the most profound susceptibility." In a more recent review Weitzenhoffer[2] has shown a summary tendency toward a skewed distribution, a larger fraction of the subjects yielding scores of low susceptibility than of high susceptibility. There are occasional findings of bimodality, with scores in the middle range being fewer than those at the extremes.[3] Because of these uncertainties it appeared desirable to repeat hypnotic susceptibility measures on a randomly selected population, and to determine the form of the distribution with more than one type of score.

Subjects and Hypnotic Procedure.—The experimental subjects were 74 undergraduate students, 40 men and 34 women, from an introductory psychology course. They volunteered for an experiment on attention, reported elsewhere.[4] At the end of this experiment they were invited to participate in an experiment involving hypnosis. Because 74 of the 78 subjects accepted the invitation, we believe that we have avoided most of the bias that would have resulted, had the students been asked in the first place to volunteer for an experiment on hypnosis.

The procedure for inducing hypnosis followed closely that proposed by Friedlander and Sarbin.[5] The subject was introduced to suggestion by experiencing the waking suggestion of falling backward from a standing position. The hypnotic procedure proper then followed. Seated in a comfortable upholstered armchair, he gazed upward at a small bright object (thumbtack) on the ceiling and received suggestions of relaxation and eye closure. After the eyes were closed (either through accepting the suggestion or by direct instruction), a number of acts were suggested, each followed by a challenge, e. g., "Your eyes are tightly shut. You cannot open them. Try to open them." By scoring successes and failures on the various performances, a score of susceptibility to hypnosis can be computed. The scoring scales used are indicated in Table 1.[6]

TABLE 1

Scoring Scales for Hypnotic Susceptibility

Test Item	Possible Score: Original Scale	Possible Score: Revised Scale
1. Eye closure	0–5	0–1
2. Eye catalepsy	0–1	0–1
3. Arm catalepsy	0–1	0–1
4. Arm rigidity	0–1	0–1
5. Hand lock	0–1	0–1
6. Verbal inhibition	0–1	0–1
7. Fly hallucination*	0–2	0–1
8. Posthypnotic suggestion	0–5	0–1
9. Amnesia	0–5	0–1
Total	0–22	0–9

* This is the only item not in the original Friedlander-Sardin scale.

Distribution of Susceptibility Based on Original Scale.—Data are also available from a University of Michigan sample of 202 subjects, previously tested on the same scale by the Weitzenhoffers.[7] The distributions for the Michigan sample and the Stanford sample are alike in their skewness, though the Stanford group proved somewhat more susceptible (Fig. 1). The reasons for the group differences cannot be ascertained but may lie in the circumstances of experimentation rather than in any differences in the population from which the samples are drawn. These results are entirely consistent with those originally presented by Friedlander and Sarbin.[5]

The Revised Scale.—The original scoring scale (Table 1) was arbitrarily weighted. It appeared to us that a study of the internal consistency of the items might suggest some less arbitrary weights. The first step was to dichotomize each of the items entering into the scale so that it could be scored on a "pass-fail" basis. It was then possible to compute tetrachoric correlations between each of the items. These are presented in Table 2. The intercorrelations are remarkably high, varying from

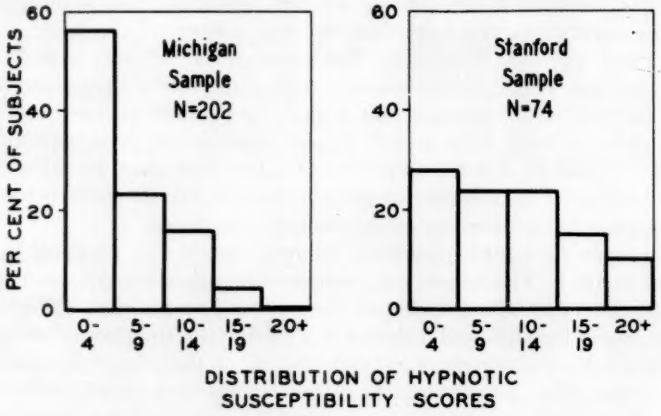


FIG. 1.

0.35 to 0.98, with a median of 0.80. A scale constructed of such items will necessarily have a high reliability. As estimated by the Kuder-Richardson formula,[8] the reliability is $r = 0.92$. Its retest reliability has to be separately determined, and was not studied in this investigation.

TABLE 2

INTERCORRELATIONS OF ITEMS ON HYPNOTIC SUSCEPTIBILITY SCALE ($N = 74$)

		Eye Clos. 1	Eye Catal. 2	Arm Catal. 3	Arm Rig. 4	Hand Lock 5	Verbal Inh. 6	Fly Halluc. 7	Post-hyp. 8
1.	Eye closure								
2.	Eye catalepsy	.65							
3.	Arm catalepsy	.78	.94						
4.	Arm rigidity	.74	.88	.95					
5.	Hand lock	.81	.91	.93	.98				
6.	Verbal inhibition	.71	.92	.95	.98	.93			
7.	Fly hallucination	.85	.73	.89	.88	.90	.76		
8.	Posthypnotic Suggestion	.35	.58	.80	.58	.80	.67	.62	
9.	Amnesia	.52	.64	66	.71	.75	.80	.83	.75

Examination of the raw data suggested that the scores might fit a Guttman-type of single-dimension scale.[9] Superficially they do. That is, if the first items are passed, the later items are more likely to be passed; after any failure, the probability of continued failure is high. Using Guttman's criteria, the index of reproducibility varies from 88 to 96 per cent for our 9 items, with a mean of 92 per cent. This is satisfactory as a fit to the Guttman-type scale.

We are very skeptical of this finding, however, in view of the processes involved. That is, if an item in a hypnotic scale is failed (a challenge is met and the suggestion is not accepted), the trance is weakened. Similarly, passing an item probably deepens the trance. Hence later items are less likely to be passed after failure and more likely to be passed after success, thus satisfying the Guttman criteria. This

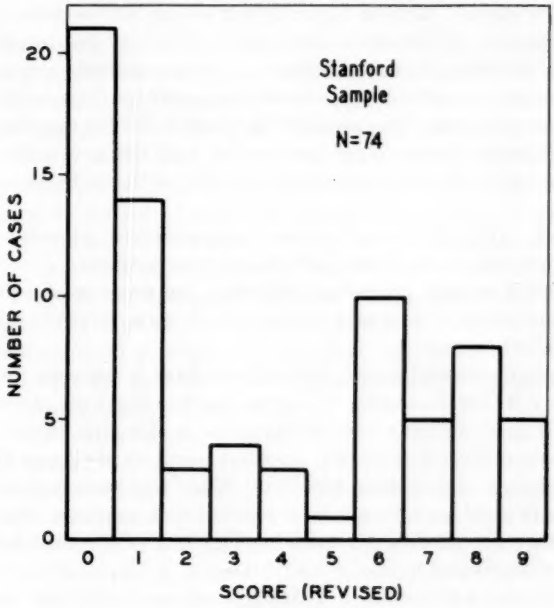


FIG. 2.

inference could be tested by using the items in various permutations. If the dimensionality held regardless of the order in which items were used, the scaling would turn out to be an artifact.

Because of the high internal consistency of the items, there appeared to be no point in assigning differential weights. Therefore, we adopted the simple scale of 1 point per item (the revised scale of Table 1). No appreciable distortion of relative position of the subjects resulted from the new scale, for scores on the original and final scale correlated to the extent of $r = +0.95$.

When the subjects were rescored on the new scale, the distribution of Figure 2 resulted. There is marked biomodality, which could have been predicted from the high intercorrelations of the dichotomized scores entering into the scale.

Sex Differences.—It has often been found that women are slightly more hypnotizable than men, but the differences, usually in the same direction, seldom reach statistical significance.[1, 7]

In our sample the mean score of 34 women on our 9-point scale was 4.6 ± 0.5 and for 40 men 2.4 ± 0.6. The critical ratio of the difference (D/σ_D) is 2.98, yielding a probability of less than 0.003 that this difference would be found if the population means for men and women were alike.

Discussion.—The fact that two sets of scores correlating + 0.95 with each other can yield distributions as different as those in Figures 1 and 2 shows the importance of extreme caution in reporting forms of distribution when the nature of the scale is unknown.

We believe that the bimodal scores of Figure 2 represent our data better than does the more orderly skewed curve of Figure 1, but we are not prepared to assert that hypnotic susceptibility is bimodally distributed. The very features that may have produced what we believe to be a spurious Guttman-type scale may also have produced the bimodality in our scores. In order to correct for this possible difficulty, a new set of instructions has been prepared in which failure is less likely to weaken the trance. Experimentation is now proceeding with this new scale. Until these data are in, we cannot be confident about the form of the distribution of susceptibility.

Summary.—1. Hypnotic induction was attempted with 40 men and 34 women who had originally volunteered for another kind of experiment.

2. The original scoring procedure, following the practice of Friedlander and Sarbin, confirmed their finding of a skewed distribution, with more unsusceptible subjects than highly susceptible ones.

3. Item analysis showed a high internal consistency between items and a reliability of $r = 0.92$ for the scale. While meeting the requirements of a Guttman-type scale, it is possible that the fit of the items to this kind of scale is spurious.

4. When a new scoring scale was adopted based on dichotomized scores for each item, a bimodal distribution resulted. While this scale represents the data better than the original scale, it cannot be asserted with assurance that the distribution of hypnotic susceptibility is bimodal. The form of the distribution is open, pending tests with revised hypnotic instructions.

5. A significant sex difference ($p < 0.003$) was found, with women more susceptible than men.

* This investigation is part of a program of research within the Laboratory of Human Development, Stanford University, aided by a grant from the Ford Foundation.

[1] C. L. Hull, *Hypnosis and Suggestibility* (New York: Appleton-Century-Crofts, 1933), p. 72.

[2] A. M. Weitzenhoffer, *Hypnotism: An Objective Study in Suggestibility* (New York: John Wiley & Sons, 1953), p. 60.

[3] F. Aveling and H. L. Hargreaves, *Brit. J. Psychol.*, **12**, 53, 1921–1922; H. J. Eysenck and W. D. Furneaux, *J. Exptl. Psychol.*, **35**, 485, 1945. While Eysenck and Furneaux argue that bimodality may result in single tests because of scales that are foreshortened at the ends, their argument is not decisive with respect to scores derived by summating dichotomized scores from several tests.

[4] A. M. Weitzenhoffer, J. Landes, and P. Gough, *J. Psychol.*, **47**, 67, 1959.

[5] J. W. Friedlander and T. R. Sarbin, *J. Abnormal Social Psychol.*, **33**, 281, 1938.

[6] The detailed instructions are available upon request but are not presented here because they are now being revised. The hypnotic sessions were conducted by the three authors assisted by Mr. Judah Landes, whose help is here gratefully acknowledged.

[7] A. M. Weitzenhoffer and G. B. Weitzenhoffer, *Am. J. Clin. Hypnosis*, **1**, 15, 1958.
[8] G. F. Kuder and M. W. Richardson, *Psychometrika*, **2**, 151, 1937.
[9] L. Guttman, in S. A. Stouffer and others, *Measurement and Prediction* (Princeton, N. J.: Princeton University Press, 1950), p. 117.

DIRECT EVIDENCE FOR THE TRANSFER OF MATERIALS FROM SYMBIOTIC ALGAE TO THE TISSUES OF A COELENTERATE

LEONARD MUSCATINE AND CADET HAND

DEPARTMENT OF ZOOLOGY, UNIVERSITY OF CALIFORNIA, BERKELEY 4

Communicated by Curt Stern, October 13, 1958

This paper takes issue with a number of inferences that have been drawn on the subject of animal-algal relationships and seeks to resolve a question that seems not to have been answered well. Specifically, it deals with the subject of unicellular symbiotic algae and their bearing on the bioeconomy of the animals which they inhabit. No attempt will be made here to summarize the literature on the subject. We refer the reader to the reviews of Buchner[1] and Yonge[2] for historical details. Attempts to delineate the role of symbiotic algae from a nutritional standpoint have resulted in a wide range of conclusions. These are (1) the animal digests whole or fragmented algal cells; (2) nutritional substances may diffuse from algae to animal, (3) there is no nutritional role whatever on the part of the algae. These conclusions are not based on direct experimental evidence; rather, they are largely drawn from circumstantial or negative evidence.

Ignoring for the present time the other important features of a balanced mutualistic association such as the exchange of gases and minerals, we have investigated the possibility that the algae do have a nutritional role and have sought to demonstrate this with direct experimental evidence. These investigations received impetus from the recent studies on coral-algal relationships by Odum and Odum[3] at Eniwetok. Their conclusions stand out in sharp contrast with those drawn by Yonge and Nicholls[4] in their now classical studies carried out on the Great Barrier Reef. Yonge and Nicholls state that "there is no evidence whatsoever of any . . . transference of material from the plants to the tissues of the animal." Contrary to this, the Odums[3] have found that, unless the nutrition of corals is regarded as partly herbivorous, the trophic structure of the coral reef community cannot be resolved. In any event, neither opinion is supported by direct evidence. A further inference in support of the Odums' viewpoint can be drawn from such studies as those of Krogh, Lange, and Smith.[5] They find that certain algae may yield up to 10 per cent of their synthesized organic matter to the external medium, which, in the instance of the coral-algal association, would be the cells of the animal. Krogh, Lange, and Smith[5] also warn that losses of organic matter to the medium may be the result of dead and decaying algal cells. More recently, Allen[6] has shown that 10–45 per cent of the organic material formed by cultures of *Chlamydomonas* appears in soluble form in the culture medium.

In considering these opposing points of view, it seemed to us that, by the use of radioisotopes and standard autoradiographic techniques, it should be possible to

demonstrate rather conclusively whether or not symbiotic algae contribute to the nutrition of the host animal.[7] Our method of attack was to place animals with symbiotic algae in sea water containing radioactive carbon dioxide. Under illumination, the algae incorporated the labeled carbon into organic molecules, whose initial location and subsequent movement was determined by autoradiographs of sections of the animal. Animals kept in labeled sea water but in the dark served as controls and indicated to us, by the same techniques, any effects independent of the algae, such as animal fixation of the radiocarbon.

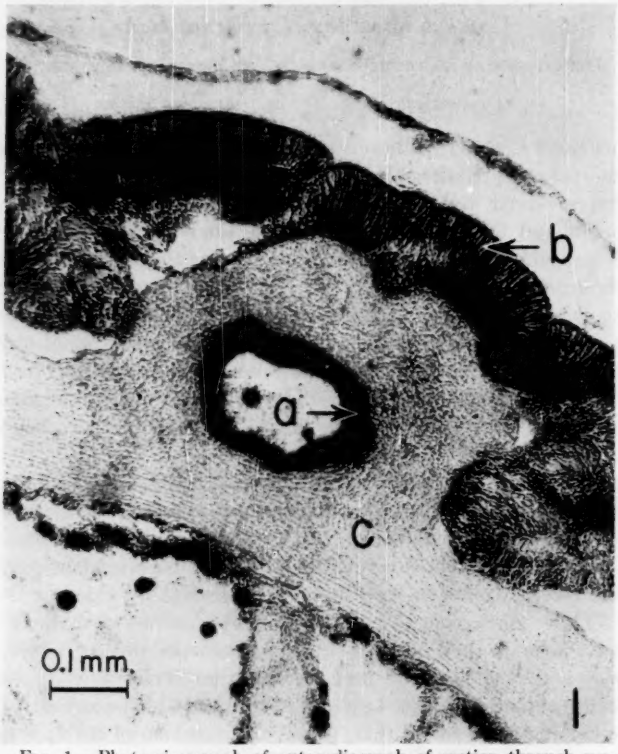


FIG. 1.—Photomicrograph of autoradiograph of section through verruca of anemone kept under illumination for 4 weeks in sea water labeled with $C^{14}O_2$. *a:* Gastrodermis and algal cells; *b:* outer epithelium; *c:* mesoglea. Photomicrographs focused to show both the section and the film exposures.

The animal chosen for this experiment was the intertidal sea anemone *Anthopleura elegantissima* (Brandt, 1835)—a small gregarious anemone which ranges from Alaska to Southern California and whose gastrodermal tissues abound with symbiotic, yellow-brown zoöxanthellae. Two millicuries of the isotope were obtained from Oak Ridge as $BaC^{14}O_3$, converted to $Na_2C^{14}O_3$, and thoroughly mixed with 2 liters of filtered, aerated sea water, resulting in a final isotopic concentration of 1 μc/ml. Twenty anemones were placed in separate 60-ml. containers of labeled sea water at 14°C; ten were held under constant illumination from two 40-watt fluorescent

tubes, and ten were kept in the dark. In both groups, five were left continuously in tagged sea water while the other five were removed after 48 hours and placed in "cold" sea water. A specimen from each group was then removed at the end of each week over a 5-week period. As a fixative, we used Carnoy's fluid to minimize the fogging effects on the film encountered with the use of aromatic fixatives. Carnoy's also dissolves out low-molecular-weight compounds, leaving only the larger organic molecules, thereby reducing the effects on the film from tagged atoms which may have diffused directly into the animal from the medium. Sections were cut

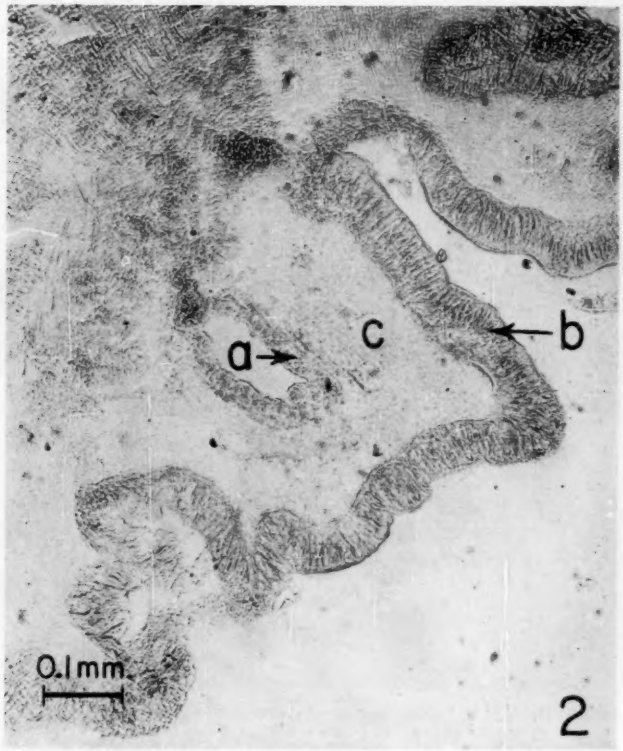


Fig. 2.—Similar section of dark control, 4 weeks. *a*, *b*, and *c* as in Fig. 1.

at 10 μ, autoradiographed with Kodak AR-10 stripping film (Kodak, Ltd., London, England), and allowed to expose the film for 9 days.

The presence of labeled material in the animal tissues of the illuminated anemones, as shown by autoradiographs of animals exposed to labeled sea water for 1–5 weeks indicated clearly that transfer from the algae had taken place. Effects independent of the algae were ruled out, in view of the absence of any tagged material in the dark controls, not withstanding some scattered exposures, not localized to any cells, which may have been the result of animal fixation. Previous experiments of shorter duration had shown that the algae incorporate enough labeled carbon over a period of 18 hours to produce a readable autoradiograph, but trans-

fer from algae to animal was not detectable. Figure 1 is a photomicrograph of an autoradiograph of a transverse section through a verruca on the column of an anemone kept in radioactive sea water for 4 weeks. The dark circular area represents exposures due to radiation from algal cells abundant in the gastrodermis. The light-gray area immediately surrounding it marks the mesoglea, only moderately labeled by comparison. The broad wavy band at the outer edge of the section delineates the outer epithelium. Judging from the density of photographic exposures, this tissue contains a moderate amount of tagged material. The thin black line beyond this represents labeled mucus. Figure 2 is a similar section of a dark control, previously described. Figure 3 is a section through the tentacular

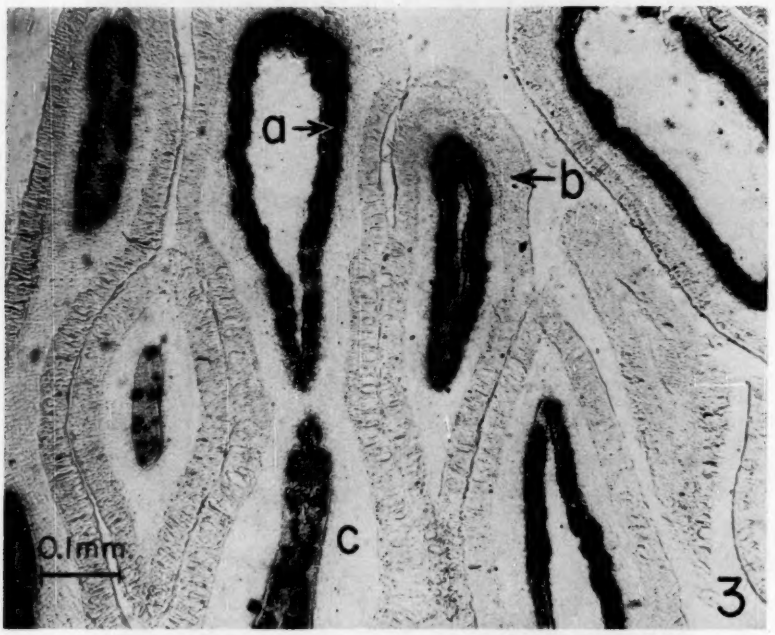


Fig. 3.—Section through tentacular region of anemone kept under illumination in labeled sea water only 18 hours. *a*, *b*, and *c* as in Fig. 1.

region of an anemone kept in labeled sea water for only 18 hours. The dark inner rings again mark the presence of algae in the gastrodermis which have incorporated labeled carbon. The relatively brief exposure time in sea water was sufficient only to effect transfer of tagged material to the gastrodermis. Mesoglea and outer epithelium are as yet unlabeled. Again, as in the 4-week dark controls, these latter tissues show scattered exposures, barely above the density of background fogging and probably due to animal fixation.

The evidence obtained from these experiments is consistent with the hypothesis that there is a transfer of material from these symbiotic algae to the host animal tissues. The probability that the same system exists in corals supports the Odums'[3] hypothesis that the algal-coelenterate complex is actually mutualistic. The recep-

tion of algal products at the higher-energy level more clearly defines the corals' role as partial herbivores. We wish to emphasize that "herbivore" is used here in the same sense as used by the Odums,[3] that of animal tissues receiving the products of algal symbionts by diffusion rather than by ingestion of particulate plant material. It should be recalled, however, that 94 per cent of the plant material in a coral colony of the type studied by the Odums[3] consists of filamentous green algae imbedded in the skeleton, the rest being zoöxanthellae. We do not wish to imply at this time, on the basis of our findings, that these filaments are involved in the same economic system; but if corals are dependent on plant symbionts for part of their nutrition, then the settlement of coral planulae in shallow, illuminated waters, the limitation of their growth to the upper photic zone, the positively phototactic orientation, and the invariable presence of zoöxanthellae in reef-building corals now appear to be more than a coincidence. Initiation, growth, and form of coral reefs must be the result of a long evolutionary history, during which adaptations favoring the association were selected for, in view of their survival value to the partners from a mutalistic nutritional standpoint.

Sea anemones with symbiotic algae have been exposed to sea water labeled with $C^{14}O_2$. By means of autoradiographs we have been able to show that after fixation of the labeled carbon by the algae there is a subsequent movement of labeled materials to all the tissues of the host anemone. In view of these results, it is suggested that the nutrition of anemones with symbiotic algae, and probably reef-building corals, is at least in part derived from materials passed to these animals as excesses of the photosynthetic activities of the symbiotic algae.

We wish to thank Professor Daniel Mazia, Department of Zoölogy, for his advice and assistance and for making the isotope and film available to us. We also extend our gratitude to Dr. Mary Belle Allen, Department of Soils and Plant Nutrition, for the loan of special apparatus used in handling the isotope. The interest of Professors Ralph I. Smith and Frank A. Pitelka, Department of Zoölogy, is gratefully acknowledged.

[1] P. Buchner, *Endosymbiose der Tiere mit pflanzlichen Mikroorganismen* (Basel, 1953).
[2] C. M. Yonge, *Biol. Rev.*, **19**, 68–80, 1944.
[3] H. T. Odum and E. P. Odum, *Ecol. Monogr.*, **25**, 291–320, 1955.
[4] C. M. Yonge and A. G. Nicholls, *Sci. Rep. Gt. Barrier Reef Exp. Brit. Mus. (Nat. Hist.)*, I, No. 7, p. 210 (1928–29).
[5] A. Krogh, E. Lange, and W. Smith, *Biochem. J.*, **24**, 1666–71, 1930.
[6] M. B. Allen, *Arch. Mikrobiol.*, **24**, 163–168, 1956.
[7] C. Hand, *Ecology*, **37**, 384–85, 1956.

THYMUS RIBONUCLEIC ACID AND EMBRYONIC DIFFERENTIATION

By M. C. Niu

THE ROCKEFELLER INSTITUTE, NEW YORK 21, NEW YORK

Communicated by Victor C. Twitty, October 6, 1958

Observations which indicated a relation between ribonucleic acid (RNA) and embryonic induction were first reported by Brachet,[1] but most recent evidence has been inconclusive.[2] It has been demonstrated, however, that the "organizer" or other embryonic tissue of urodeles, when explanted into hanging drops of a balanced salt solution, releases diffusible substances capable of initiating differentiation of the young gastrula ectoderm.[3] Analysis of the medium withdrawn from such cultures suggested that the active component may be RNA.[4] Studies with RNA isolated from mammalian organs have produced evidence of its capacity to cause differentiation and that the RNA may possess inductive specificity.[5] When presumptive ectoderm cells were cultured in salt solution containing an appropriate concentration of RNA, freshly isolated from various sources, variations in the pattern of differentiation resulted. For example, some cultures treated with RNA from calf kidney had the appearance of tubule-like condensations. Liver RNA induced the formation of a morphological type not observed previously in any series employing the diffusible substances mentioned above, or kidney RNA. In the extension of these experiments, cultures with specific patterns were implanted into the upper flank of the posterior trunk of advanced embryos (stage 30–34). Later it was found that some implants had differentiated into structures which resembled embryologically the tissue source of the RNA.[6,7]

In this communication evidence is presented which suggests that extracts obtained from calf thymus induced specific differentiation and that the active component of the extracts is RNA.

EXPERIMENTAL

Preparation of Samples.—Calf thymus was obtained from the slaughterhouse. When collected, it was put immediately into ice-cold sucrose solution (0.25 *M*). All preparations were carried out in the cold room at 0–2° C. Thymus tissue (100 gm.) was finely chopped and homogenized with 400 ml. 0.9 per cent NaCl at top speed in a Waring Blendor for 1 minute. The homogenate was filtered through 2 layers of gauze and stirred for 1 hour. During stirring, the pH was brought to 7.8 by adding 1N NaOH drop-wise. The filtrate was centrifuged at 5,000 r.p.m. for 30 minutes. The sediment was discarded and the supernatant fluid divided into two portions. To one portion, 2 volumes of 95 per cent ethanol were added, and the mixture was centrifuged. The sediment was resuspended in saline and the pH adjusted to 7.2. After stirring for 20 minutes, the preparation was centrifuged at 8,800 r.p.m. for 1 hour. The supernatant solution was precipitated twice more, and the sediment redissolved in saline. Undissolved substances, if present, were removed by centrifugation. The supernatant fraction is designated as the "saline extract" (SE).

The pH of the other portion was carefully lowered by 1 N HCl to pH 4.5 and centrifuged. The sediment was resuspended in saline, and the pH raised to 7.2. After stirring for 30 minutes the undissolved particles were removed by centrifuga-

tion. The supernatant fluid was either precipitated twice, as mentioned in the preparation of SE, or dialyzed on a rocker overnight, against running distilled water, and subsequently centrifuged at 8,800 r.p.m. for 1 hour. To the supernatant fraction, potassium acetate was added up to 2 per cent, followed by 2 volumes of ethanol. The mixture was centrifuged. The sediment was resuspended in saline, and the pH adjusted to slightly above neutrality. The precipitate, if any, was removed by centrifugation. This supernatant fluid is referred to as the "ribonucleoprotein fraction" (RF).

For the isolation of RNA, the procedure adopted was that of Kirby,[8] with some modifications. The filtered homogenate mentioned above was stirred while adding an equal volume of 90 per cent phenol (wt/v) in room temperature (24° C.). Stirring was continued for 1 hour, and the mixture was then centrifuged at 1,800 r.p.m. in the cold room for 30 minutes. The top aqueous layer containing RNA was removed by suction. (For a greater yield, the phenol layer should be washed once or twice with saline, and the washings should be combined with the original aqueous fluid. Since our major object has been rapid extraction rather than maximal yield, this step has been omitted.) Potassium acetate was added to the aqueous solution up to 2 per cent, and then 2 volumes of alcohol were added. The sediment was removed by centrifugation and redissolved in the modified Holtfreter solution[3] (referred to henceforth as the "salt solution"). The RNA solution was either reprecipitated three times or dialyzed against running distilled water overnight in the cold, and centrifuged at 20,00 r.p.m. for 30 minutes or at 8,800 r.p.m. for 1 hour. To the supernatant fluid, potassium acetate was added up to 2 per cent, then mixed with 2 volumes of alcohol and centrifuged. The sediment was dissolved in the salt solution.

Chemical Analysis.—UV absorption spectra of the samples were measured with the Beckman spectrophotometer. The solution of RF was analyzed by zone electrophoresis as described by Taylor *et al.*[9] The starch was thoroughly washed with phosphate buffer (pH 7.1, 0.1 *M*) in order to remove nitrogen-containing substances which might contaminate the preparation. Thymol was added as a preservative. The solution of R F was applied to a starch block 70 cm. long, 4 cm .wide, and 1 cm. deep. Electrophoresis was carried out with phosphate buffer at 0°–2° C., and 300 volts were applied across the electrodes for 20 hours. After electrophoresis, the starch block was cut into 2-cm. segments, and each was extracted with 4 ml. of water. The nucleic acid was located by means of its absorption at 260 mμ and the protein by absorption at 280 mμ.

Dry Weight.—The sample was precipitated by alcohol, washed once with 95 per cent alcohol, and once with absolute alcohol. After centrifugation, the alcohol was decanted. The test tubes containing the precipitates were placed in an inverted position for 1 hour, and then dried at 110° C. overnight.

Nitrogen and Phosphorous.—After Kjeldahl digestion of the samples, the nitrogen content was determined by direct nesslerization, and the phosphorous content by Allen's method.[10]

RNA.—A sample was precipitated by alcohol and washed once with cold 2 per cent perchloric acid (PCA). The sediment was heated with 10 per cent PCA at 70° C. for 20 minutes to extract the nucleic acid. The RNA content was estimated

by the ultraviolet extinction coefficient of the PCA extract. Protein N was determined after the removal of RNA.

Enzymatic Treatment.—RNase, trypsin, and/or chymotrypsin were used. RNase was purchased from Worthington, but tests with GBI and Armour products were also made. The trypsin was from Worthington. We appreciate the generosity of Dr. Kunitz, of the Rockefeller Institute, for supplying twice-crystallized chymotrypsin. These enzymes were dissolved in saline (pH 7.4; concentration 1 mg/ml). One ml. enzyme and 9 ml. SE or RF (in saline pH 7.4 or salt solution pH 7.8) were incubated at room temperature (25° C.) for 90 minutes. After incubation, the enzymatic action was stopped in the cold by adding an equal volume of 4 per cent perchloric acid, and mixing thoroughly. Twenty minutes later, the mixtures were centrifuged at full speed of a clinical centrifuge for 5 minutes. UV absorption of the supernatant solution of the RNase-treated sample was determined at 260 mμ, and of those treated with proteolytic enzymes at 280 mμ. Both the RNA and the protein N of the sediments were determined as above.

RNase hydrolysis was also carried out for 6 hours, followed by dialysis on a rocker against running distilled water overnight. One-third of the dialyzed samples was washed with an equal volume of cold 4 per cent perchloric acid. The precipitate was analyzed for RNA. To the other two-thirds, an equal volume of cold, saturated ammonium sulfate was added and mixed well. Thirty minutes later the mixture was centrifuged. In order to remove the ammonium sulfate, both fractions were again dialyzed. Afterward, the RNA content was estimated.

In one series, "tris" (tri(hydroxymethyl) aminoethane) buffer, 0.05 *M*, pH 7.4, was used. RNase was dissolved in water (1 mg/ml). Five ml. of the sample plus 5 ml. of "tris" buffer, 0.1 *M*, pH 7.4, and 1 ml. enzyme were incubated for 90 minutes at room temperature. The enzymatically split products and the RNA content were estimated as above.

Embryological Tests.—A known amount of the sample was dissolved in the salt solution and used to make hanging-drop cultures of young ectoderm from gastrulae of *Amblystoma tigrinum* (stage 10). The volume of each culture was about 0.01 ml. The size of the ectodermal explants (approx. 50 cells) was about one-sixtieth of the total presumptive ectoderm of an early gastrula. The cultures were kept 10–16 days at 19°–20° C.

At the end of the culturing period, half were fixed for whole mounts and the others were freed, using the tip of forceps, for implantation. The liberated culture was implanted between the epithelial and mesodermal layers of young larvae (stages 30–34). The implants were uniformly located on the posteriodorsal flank, at the level of the yolk border of the posterior trunk. After a few weeks, the larvae were fixed in Bouin's solution. Serial sections were cut 8 μ in thickness and stained with hematoxylin and eosin.

RESULTS

Chemical Analysis.—Both fractions SE and RF, exhibited UV absorption spectra characteristic of RNP. Zone electrophoresis showed that the proteins and RNA of RF moved as a united front (Fig. 1). This does not indicate, however, that these two components were necessarily combined as RNP in the cells; artificial combination during the process of isolation is not excluded.

The analytical data on these two samples are given in Table 1. RF has a nitrogen-phosphorus ratio of 5.4 and contains 35 per cent RNA; SE has a ratio of 7.6 and contains 8 per cent RNA. While RNA-P accounts for all phosphorus of RF, that in SE constitutes only half the amount, indicating the presence of other P-containing, acid-insoluble materials.

The RNA had maximal and minimal extinction coefficients at 258 and 230 mμ, respectively. The ratio between the maximum at 258 and the minimum at 230 was about 2.2. The nitrogen-phosphorus ratio was around 1.78. The orcinol test for RNA was positive, yielding a green color. Biuret and iodine tests were negative.

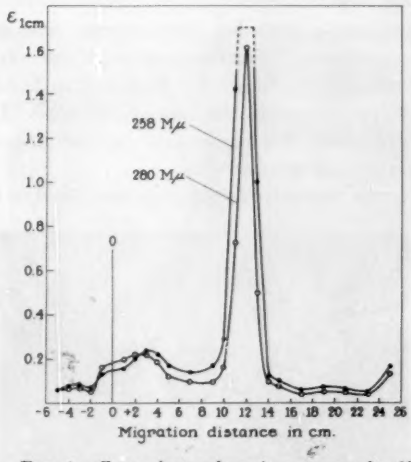


FIG. 1.—Zone electrophoresis pattern of calf thymus RF after 20 hours' migration through starch in phosphate buffer (0.1 *M*), pH 7.1. Nucleic acid distribution is indicated by E_{258}, and protein distribution is shown by E_{280}.

Inductive Activity.—In dealing with physiological function, the concentration of the active material is of prime importance. For practical purposes, the RNA contents of SE and RF were estimated by their optical densities. The optimum biological activity was found at concentrations having $E^{1\text{ cm}}_{260\text{m}\mu}$ between 0.6 and 1.0 (about 30–50 μg. RNA/ml). To achieve this concentration range with the samples mentioned above, SE can be diluted 6–10 times and RF 14–23 times. At these dilutions, SE contains 0.36–0.6 mg. and RF 50–100 μg/ml. of total extracted substances. Each of the hanging-drop cultures contains, therefore, 0.3–0.5 μg. RNA, and 0. 5–6 μg. total extracted material.

Young ectoderm of *A. tigrinum* was explanted into the hanging drops of the salt solution (control) and into the salt solution containing SE or RF. In the control series the ectodermal explants became attached earlier and usually spread into a thin sheet. Retraction of the sheet occurred frequently. An analysis of the development showed that 50 per cent of the cultures became epithelial sheets or balls, 0–15 per cent produced nerve fibers with occasional pigment cells, and the rest disintegrated. A great majority of the explants of the experimental series gave rise to differentiated outgrowths. Morphologically, there were two distinct types (Table 2). One shows neural differentiation, and the other is clearly non-neural but possesses a specific morphological pattern. Compared with the neural formation in the controls, the outgrowths of the experimental series were luxuriant and

TABLE 1

COMPOSITION OF THE THYMUS EXTRACT

Samples	Dry Weight (mg./ml.)	Nitrogen (mg./ml.) (Per Cent)	Phosphorus (mg./ml.) (Per Cent)	N/P	RNA-P (mg./ml.)	RNA (Per Cent)
SE	3.9	0.46 (11.7)	0.06 (1.5)	7.6	0.03	8
RF	2.0	0.37 (18.5)	0.07 (3.5)	5.4	0.07	35

TABLE 2

INDUCTIVE CAPACITY OF THE SALINE EXTRACT

Series of Experiments	RNA Content (Mg./Ml.)	Neural Differentiation	Non-neural Differentiation	No Differentiation	Per Cent of Induction
1	31	26	12	12	76
2	61	23	13	14	72
3	54	32	4	14	72
4	27	26	3	11	73
Average	43	27 (56%)	8 (17%)	13 (27%)	73
Control	0	0–7	0	43–50	0–15

frequently included mesenchyme cells and chromatophores, in addition to the nerve fibers. The composition of the non-neural cultures varied somewhat but can be shown by Figure 2. As shown in Table 2, neural differentiation averages about 56 per cent and the non-neural type 17 per cent. Furthermore, the frequency with which the non-neural cultures appear can be increased fourfold (see section on RNase treatment).

The non-neural culture is composed of three elements: (1) an epithelial thicken-

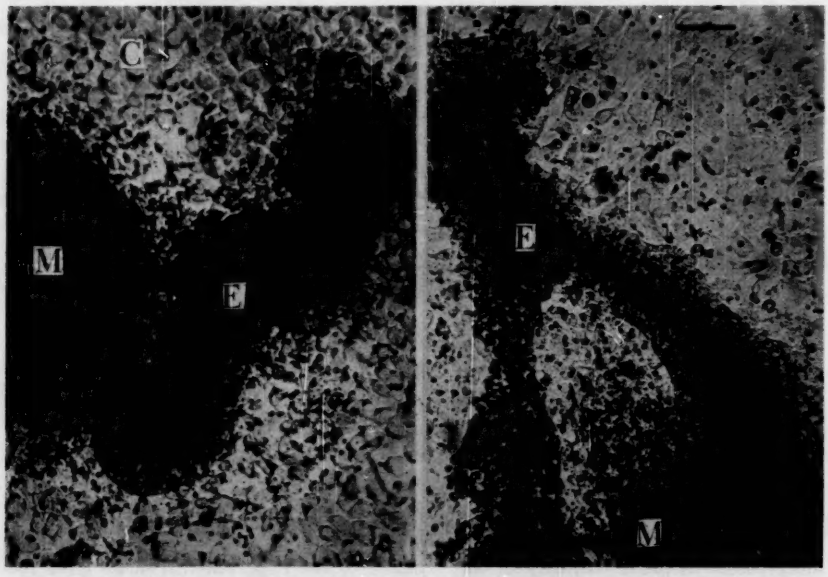
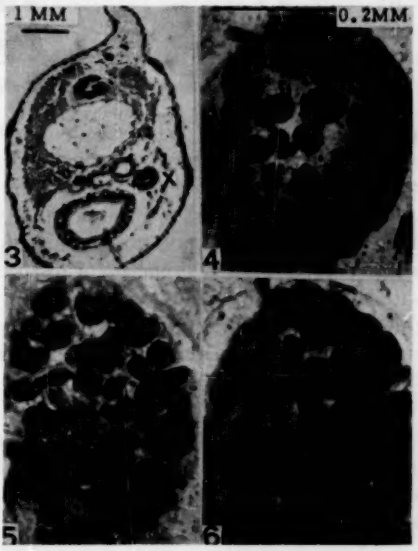


FIG. 2.—Two photomicrographic pictures show variations in arrangement of the three components of two living, non-neural cultures (grown in thymus SE for 14 days). *C*, connective tissue cells; *E*, epithelial thickening; and *M*, mesodermal mass.

ing, (2) a mesodermal mass, and (3) connective tissue cells which constitute the main portion of the outgrowth. The three components are distinct and independently developed. Originally, the ectodermal explant attaches itself firmly to the substratum (glass). This phase is followed by a continuous, radial migration of cells from the explant. These become the connective tissue cells. At the same time, the explant itself spreads out in various forms, such as patches or tongue-shaped protrusions. The latter develop into the epithelial thickenings, while the

remaining original explant transforms into the mesodermal mass. The smaller masses undergo the most extensive reduction in thickness. In those which have been reduced to monocellular layers, fibrous strands develop.

Implantation of Cultures.—Occasional control implants differentiate into small masses of nerve tissue, but usually the implanted control cultures disappear and cannot be relocated after several weeks of implantation. Apparently, the cells become incorporated with the host tissue. The implant of a well-developed non-neural culture from the experimental series is shown in Figure 3. In this case the implanted culture has formed a small compact body. It is more or less spherical and has a sheath. In the matrix there are two kinds of nuclei: spherical and ellipsoidal (Fig. 4). These nuclei resemble those of host thymus body in appearance (Fig. 5). They differ from host ganglia. The ganglion of a young larva does not have a recognizable sheath, and its nuclei are uniformly spheroidal (Fig. 6).



FIGS. 3–6.—Cross-section of a young larva, showing a small compact body (*X*) developed from the implant of a non-neural culture (18 days after implantation). Figs. 4, 5, and 6 are photomicrographs with the same magnification. Fig. 4 shows the cross-section of the small body (*X*) in Fig. 3; Fig. 5 shows the cross-section of a thymus body of *Amblystoma punctatum* larva; and Fig. 6 shows the cross-section of a cranial ganglion.

Enzyme Analysis.—Analysis of the active principle in SE and RF has been based on the correlation between partial removal of known constituents and the resultant loss of inductive capacity of the residual material. This was accomplished with enzymes RNase, trypsin, and/or chymotrypsin. It is necessary to determine the extent of enzymatic digestion of the substrate by estimating either the amount of substances rendered soluble in acid or the remaining substrate. The data in Table 3 show that proteolytic enzymes removed 26–29 per cent of the protein N of SE, and 54–59 per cent of the protein N of RF. RNase removed 63 per cent of RNA of SE, and 62.4 per cent of RNA of RF. Various methods were employed in attempts to increase the removal of RNA from SE or RF. Prolonged hydrolysis by RNase increased the removal of RNA only slightly up to 71 per cent (Table 4). The ammonium sulfate–precipitated sediment of the RNase-treated sample was found to have approximately half the total amount of RNA present in the sample before the ammonium sulfate treatment. Finally, with lyophilized RNP (which was non-inductive), only 79–81 per cent of the RNA content was removed by RNase (Table 5). It should be noted that an appreciable amount of RNA remained in each instance, although the methods are considered to be maximally effective for its removal.

The split products of the enzyme-treated materials were removed by dialysis

TABLE 3

ENZYMATIC HYDROLYSIS OF SE AND RF*

Samples	Extract		Trypsin	Chymotrypsin	Control	Ribonuclease
SE	2% PCA (acid-soluble substances)	O.D. at 260 mμ	....	....	0.215	0.390
		280 mμ	0.243	0.225	0.182	....
	10% PCA, 70° C. 20 min. (RNA) O. D. at 260		0.550	0.476	0.433	0.159 (−63%)
	Protein N (mg./ml.)		0.0159 (−29%)	0.0166 (−26%)	0.0223	0.0213
RF	2% PCA	O.D. at 260	....	....	0.220	0.425
		280	0.255	0.225	0.116	
	10% PCA O.D. at 260		0.624	0.543	0.615	0.231 (−62.4%)
	Protein N (mg./ml.)		0.0302 (−54%)	0.0332 (−59%)	0.0562	0.0582

* Incubated at 25° C., 90 minutes. O.D. = optical density.

against salt solution. When treated with trypsin, the remaining, unhydrolyzed portion had lost all inductive ability. Since proteolytic enzymes removed only 26–59 per cent of the protein, the complete biological inactivation of the remaining protein was unexpected.[11] One possibility might be that the added enzyme may have complicated the situation. This is particularly likely with trypsin, because of its well-established property of dissociating cells. Its presence may interfere with the reactivity of the tissue to such an extent that differentiation fails to occur. One means of stopping trypsin action is to inactivate it by adding an equimolar concentration of soy bean inhibitor. Experiments carried out with samples thus treated showed inductive activity. Soy bean inhibitor alone had no inductive capacity.

RNase-treated samples retain inductive activity, but at a reduced level. In one series of experiments (Table 2, No. 1), for example, following RNase treatment, the sample had 18 μg. RNA/ml and gave rise to 14 non-neural cultures, 18 neural, and 18 without differentiation. A loss of 40 per cent (13/31) RNA resulted in a

TABLE 4

EFFECT OF RNASE ON SE AND RF*

SE	Acid-soluble substances (O.D. at 260 mμ)	Control	0.230
		Explant	0.208
	RNA (O.D. at 260 mμ)	Control	0.432
		Explant	0.128 (−71%)
RF	Acid soluble substances (O.D. at 260 mμ)	Control	0.330
		Explant	0.288
	RNA (O.D. at 260 mμ)	Control	0.511
		Explant	0.152 (−70%)

* Incubation 6 hours at 25 °C. and followed by dialysis, on rocker, against running distilled water. O.D. = optical density.

TABLE 5

ENZYMATIC STUDIES ON LYOPHILIZED pH 7.1 RNP OF THYMUS NUCLEI*

		O.D. at	Trypsin	Chymotrypsin	Control	RNAse
In Saline, pH 7.3	Acid-soluble substances (1/4 concentration)	260 mμ	0.424	0.513	0.265	0.380
		280	0.388	0.461	0.204	0.258
	RNA	260	0.759	0.697	0.981	0.191 (−81%)
In tris Buffer 0.05 *M* pH 7.4	Acid-soluble substances (1/4 concentration)	260	0.250	0.228	0.210	0.320
		280	0.188	0.162	0.143	0.229
	RNA	260	0.512	0.515	0.598	0.128 (−79%)

* Incubated at 25° C., 90 minutes. O.D. = optical density.

15 per cent (6/38) reduction of activity. It was surprising to note that the development of nonneural cultures increased slightly. The RNA concentration of the RNase-treated sample, just mentioned, was adjusted to the level of the non-treated sample. If reduction of RNA were actually specifically responsible for the loss of 15 per cent activity, restoration to the original concentration should result in returning the lost activity. The actual finding was 74 per cent (32 non-neural cultures, 5 neural, and 13 without differentiation). This agrees with the average 73 per cent obtained from SE (8 non-neural, 27 neural, and 13 with no differentiation). The significant point is the fourfold increase of non-neural cultures. The increase is apparently associated with the undigested portion of the RNA component.

The Role of RNA in Differentiation.—Freshly isolated RNA (40 μg/ml) was used for ectodermal differentiation. It gave rise to 3 non-neural cultures, 14 neural, and 33 without differentiation. Because of the low frequency in induction, the activity was further checked. This was done by mixing a foreign protein (soy bean inhibitor) with RNA. The concentration was adjusted to 40 μg. RNA and 100–300 μg. inhibitor per ml. The result of the activity test was 9 non-neural cultures, 20 neural, and 21 without differentiation. Thus the activity climbed from 34 to 58 per cent. Since soy bean inhibitor is not an inductor, this finding indicates that the protein plays a role in differentiation but is not causative. RNA appears to be the causal agent.

DISCUSSION

The biological activity of the inductor substances has been expressed in terms of structures developed at given concentrations. Zero to 15 per cent of the cultures grown in the salt solution (control) gave rise to neural derivatives. An inductor substance should produce a higher frequency of neural structures, and/or other distinct morphological types. As demonstrated in Table 2, the extract of thymus has given not only a higher frequency of neural derivatives but also non-neural structures that have never been observed to develop either in the salt solution or from cultures with extract of liver or kidney.[5,6] The formation of two types of cultures suggests that the sample may contain both a nerve-forming factor and another factor responsible for the genesis of structures specific to the thymus.

Chemical analysis has shown that both RF and SE contain large amounts of RNP. Since RNP appears to cause embryonic differentiation,[4, 12–14] one might assume that the active principle of the thymus extract may also be RNP. However, there is no evidence, so far, to show that non-RNA, phosphorus-containing substances of SE do not play a role. The first series of experiments on the chemical nature of the active substances involves enzyme studies using RNase, trypsin, and/or chymotrypsin. Proteolytic enzymes were found to remove 26–59 per cent of the protein with loss of inductive capacity. Subsequently, it was shown that inactivation of the trypsin by equimolar concentration of soy bean inhibitor restored the activity. Thus the loss of activity was due to the presence of trypsin rather than to inactivation of the unhydrolyzed materials.

Inactivation of trypsin was also achieved by heating at 85° C. in 95 per cent alcohol for 5 minutes.[15] Experiments with the trypsin-treated RNP showed a progressive reduction of its inductive activity. Hayashi ascribed the reduction

of activity directly to the loss of protein. In the same experiment, however inactivation (denaturation) of some proteins of the RNP did not result in a loss, in inductive activity. Analysis of his results suggests that the inductive activity may have resided in the inactivated or denatured proteins or in the substances still soluble following heating in alcohol. The inductive activity of protein denatured by phenol extraction has been demonstrated by Tiedemann and Tiedemann.[16] Activity of this nature could not be involved in the present results, however, since denatured proteins do not dissolve in water and saline and, accordingly, cannot be tested in our system.

The effect of RNase on SE or RF varied somewhat with the sample, the condition of storage, and the commercial source of the enzyme. At best, the hydrolysis we obtained was 71 per cent of the RNA component.[17] Usually it was about 63 per cent and sometimes only 40 per cent. As the result of a loss of 40 per cent RNA in the sample, a reduction in differentiation capacity of 15 per cent was demonstrated. There is a report that the sedimentable and non-sedimentable fractions of streptomycin-precipitated RNP contain, respectively, 10.2 and 92.7 μg RNA-P/mg protein N. Their frequency of induction was 72 and 100 per cent.[14] The relationship between RNA content and inductive capacity is not unlike the results reported herein.

The above considerations lead to the conclusion that embryonic differentiation is initiated by the RNA component of SE and RF. Direct evidence for the role of RNA in differentiation is provided by studies with RNA freshly isolated from the thymus. It should be noted that the isolation procedure we adopted was simple and fast. The material was neither dried nor lyophilized. If allowed to dry, RNA becomes inactive.[18, 19] With the optimal concentration, the inductive frequency of RNA was 34 per cent and increased to 58 per cent when mixed with a non-inductive, foreign protein. Apparently, the role of RNA in differentiation is comparable to that in the infectivity of tobacco mosaic virus (TMV).[20] RNA and the protein components of TMV could be dissociated and reconstituted successfully.[21] When RNA of stain A is reconstituted with protein of strain B and inoculated into the plant leaves, the resulting TMV contained protein of strain A instead of B.[22] Recently, TMV-RNA was found to be as infectious as the intact TMV when reconstituted with non-infectious protein.[23] Apparently, the protein helps manifest the functional potentiality of RNA. Although the mechanism by which the protein operates is not clear, it may act as a stabilizer, a carrier, or an agent to facilitate the entrance of RNA into the cells.

It was shown that the average frequency of differentiation induced by SE was 73 per cent, and 58 per cent by the mixture of RNA and foreign protein. The closeness of the two percentages is taken as an indication that the activity of both preparations is due to RNA, because the foreign protein of the mixture is non-inductive. Particularly striking are the data regarding the non-neural differentiation (17 per cent by SE and 18 per cent by the mixture of RNA and protein). Experiments with RNase-treated samples have shown further that the unhydrolyzed "core" of the RNA component gives rise to a higher frequency of non-neural cultures (see p. 1271). The hydrolyzable portion could be considered as "masking" the capacity for the non-neural differentiation. It appears that the nerve-forming factor and the factor for the genesis of non-neural structures are

indeed substances of one category. This category of substances appears to be the ribonucleic acid extracted from the constituent cells of the organ. Several RNA components are known to exist in the cell. There are two in the nucleus—the nucleolar RNA and the chromosomal RNA. The cytoplasm contains microsomal RNA and the soluble RNA. Each of these may possess a definite activity. The soluble RNA of the liver, for example, has been shown to be the "carrier" of ATP-activated amino acid to the microsome, whereas the microsomal RNA, with the co-operation of guanosine triphosphate, transfers the activated amino acid to peptide linkage.[24]

The composition of the culturing medium is known to affect the morphology of the culture.[25] The possibility should be considered that the formation of non-neural structures is a form of "modulation." The ectodermal cells, unlike adult tissue, are provided with nutrient stores and thus do not need any exogenous, nutritional support. The amount of RNA used to bring out the distinctive structure is very small. These two considerations are in contrast to the conditions calling for "modulation." Furthermore, when non-neural cultures were implanted into the flanks of young larvae, they developed into small bodies resembling the host thymus.

SUMMARY

Saline extract (SE) and ribonucleoprotein fraction (RF) were prepared from calf thymus. The data from chemical analyses varied somewhat according to the preparation. The samples reported here contain, respectively, 8 and 35 per cent RNA, and their nitrogen-phosphorus ratios are 7.6 and 5.4, respectively. Zone electrophoresis of RF indicated that the protein and RNA are combined as ribonucleoprotein.

Both SE and RF were used to cultivate small explants of the presumptive ectoderm of *A. tigrinum* in hanging drops. The extracts employed contained 30–50 μg RNA/ml. While the control series gave rise to 0–15 per cent of cultures showing neural differentiation, SE and RF induced the formation of a much greater percentage (73) of the neural and non-neural structures. Neural differentiation includes the appearance of nerve fibers with or without the outgrowth of mesenchyme cells and chromatophores. Three components are identifiable in the non-neural culture—an epithelial thickening, mesodermal mass, and connective tissue cells.

Trypsin and chymotrypsin removed about 26–59 per cent protein N, and RNase removed 40–71 per cent RNA. After trypsin treatment, the sample became inactive. This was due to the presence of enzyme in the sample rather than to the inactivation of the unhydrolyzed portion. The reduction of RNA content after RNase treatment has been correlated with the loss of the inductive ability. When the RNA content of the RNase-treated SE was increased to the level of the untreated, the inductive activity (74 per cent) was found to be within the range of the untreated (73 per cent). 64 per cent of the cultures were non-neural. It appears that the undigested "core" of the RNA component of SE is responsible for the specificity which the hydrolyzable portion tends to mask.

The correlation between the amount of RNA and the frequency of embryonic differentiation was shown also by the experiments using chemically defined RNA.

Like SE and RF, RNA was capable of inducing both neural and non-neural structures, but at a comparatively lower frequency. When it was mixed with a foreign, noninductive protein, the inductive activity increased almost to the extent characteristic of SE or RF.

It is a pleasure to acknowledge the constant aid and interest of Dr. Alfred E. Mirsky, in whose laboratory the experiments were performed, and to thank Miss Carol Costello for her expert technical assistance.

[1] J. Brachet, *Embryologie chimique* (Paris: editions Desoer, Masson et Cie, 1947).
[2] J. Brachet, *Biochemical Cytology* (New York: Academic Press, 1957).
[3] M. C. Niu, and V. C. Twitty, these PROCEEDINGS, **39**, 985, 1953.
[4] M. C. Niu, in *Cellular Mechanisms, Differentiation and Growth, ed.* D. Rudnick (Princeton, N.J.: Princeton University Press, 1956), p. 155.
[5] M. C. Niu, A.A.A.S. Symposium paper, December, 1956 (in press).
[6] M. C. Niu, *Anat. Rec.*, **131**, 585, 1958.
[7] M. C. Niu, unpublished data.
[8] K. S. Kirby, *Biochem. J.*, **64**, 405, 1956.
[9] S. P. Taylor, V. DuVigeaud, and H. G. Kunkel, *Biochem. J.*, **205**, 45, 1952.
[10] R. J. L. Allen, *Biochem. J.*, **34**, 858, 1940.
[11] T. Yamada and K. Takata, *Exptl. Cell. Res.*, suppl., **3**, 402, 1955.
[12] Y. Hayashi, *Embryologia*, **3**, 57, 1956.
[13] T. Yamada and K. Takata, *Embryologia*, **3**, 69, 1956.
[14] T. Yamada, *Experimentia*, **14**, 81, 1958.
[15] Y. Hayashi, *Embryologia*, **4**, 33, 1958.
[16] H. Tiedemann and H. Tiedemann, *Z. physiol. Chem.*, **306**, 7, 1956.
[17] Y. Hayashi, *Embryologia*, **2**, 145, 1955.
[18] T. Yamada, K. Takata, and S. Osawa, *Embryologia*, **2**, 123, 1954.
[19] H. Tiedemann and H. Tiedemann, *Z. physiol. Chem.*, **306**, 132, 1956.
[20] A. Gierer and G. Schramm, *Nature*, **177**, 702, 1956.
[21] H. Fraenkel-Conrat and R. C. Williams, these PROCEEDINGS, **41**, 690, 1956.
[22] H. Fraenkel-Conrat, *J. Am. Chem. Soc.*, **78**, 882, 1956.
[23] W. D. Takahashi, reported at AIBS meetings in Bloomington, August, 1958.
[24] M. S. Hoagland, M. L. Stephenson, J. F. Scott, L. T. Hecht, and P. C. Zamecnik, *J. Biol. Chem.*, **231**, 241, 1958.
[25] T. T. Puck, in *Rhythmic and Synthetic Processes in Growth*, ed. D. Rudnick (Princeton, N. J.: Princeton University Press, 1957), p. 1.

---

*CARTILAGE FORMATION IN SOMITE GRAFTS OF CHICK BLASTODERM**

BY TAMIKAZU SENO AND ILHAN BÜYÜKÖZER

DEPARTMENT OF ANATOMY, GUNMA UNIVERSITY, MAEBASHI, JAPAN, AND INSTITUTE OF HISTOLOGY AND EMBRYOLOGY, ANKARA, TURKEY

Communicated by R. K. Burns, September 19, 1958

INTRODUCTION

Results obtained from the transplantation of isolated somites of the chick embryo have not been consistent in regard to the capacity of the somite mesoderm for independent differentiation or self-differentiation. In 1954 Watterson *et al.*[1] reported that groups of several somites isolated from the neural tube and notochord

of embryos possessing 17–28 pairs of somites, approximately 2 days' incubation age, were incapable of forming cartilage in chorio-allantoic grafts. Similar isolates from older, 3-day embryos having 35–38 somites produced cartilage but in relatively small amounts. By the fourth day of incubation the cartilage-forming capacity of the somite was well established, and large masses of cartilage appeared in the grafts. Thus it was suggested that the differentiation of cartilage is dependent upon the presence of the neural tube and notochord until relatively late in development, i. e., until approximately the third day of incubation. Confirming evidence was obtained by including the adjacent neural tube and notochord with isolates of 2-day somites which alone had failed to form cartilage. Under these conditions, cartilage now differentiated in the grafts. Essentially similar observations were made by Avery *et al.*[2] and Lash and Holtzer[3] from tissue culture and chorio-allantoic grafting studies. On the basis of such findings, it has been postulated that the neural tube and notochord play an inductive role in the formation of axial skeletal tissue.

Murray and Selby,[4] on the contrary, found that cartilage, bone, and skeletal muscle would differentiate in chorio-allantoic grafts of somites isolated from the neural tube and notochord of 2-day embryos. Furthermore, paraxial mesoderm, prior to its segmentation into somites, isolated similarly from embryos slightly younger, reacted in the same manner. More recently Straus and Rawles[5] obtained both cartilage and striated muscle from intra-coelomic grafts of somites isolated from the neural axis of embryos having 25–28 pairs of somites.

The difference in results between the two groups of investigators is undoubtedly related to differences in the methods of isolation of the somite mesoderm. Murray and Selby[4] and Straus and Rawles,[5] in contrast to those mentioned earlier,[1–3] did not remove the associated ectoderm and endoderm from the somite mesoderm. Hence the isolate was composed always of three germ layers—ectoderm, mesoderm, and endoderm—in their normal relationships, indicating that the normal spatial tissue relationships play an important role in the differentiation of cartilage and skeletal muscle.

The following experiments were therefore designed primarily to determine whether somite mesoderm isolated from the neural axis of still earlier somite stages (8–20 somites), not heretofore investigated, would differentiate into cartilage and skeletal muscle in grafts, if the spatial relationships between the mesoderm and its associated ectoderm and endoderm remained undisturbed. Furthermore, by using donor embryos in early somite stages, it would be possible to remove somites prior to the entrance of migratory neural crest cells and thus to ascertain whether or not crest cells of the trunk region are necessary for the fomation of cartilage.

MATERIALS AND METHODS

The developmental capacities of the somite mesoderm were tested by (1) grafting to the embryonic coelom of host embryos of 60–65 hours' incubation and (2) grafting to the chorio-allantoic membrane of 8–9-day host embryos. (A detailed description of both methods may be found in Hamburger's *Manual of Experimental Embryology*.[6]) Eggs of White Leghorns were used chiefly for donor and host material, but in some cases the donor embryos came from eggs of a pigmented

breed, the Silver Campine. The preparation of the transplant was essentially the same for both methods of transplantation and was briefly as follows: The eggs were incubated at a temperature of 37.5°–38°C. for 30–40 hours, during which time embryos having from 8 to 20 pairs of somites were usually obtained. Older embryos up to 30 somites were also used for comparison in some of the experiments. The donor blastoderm was removed from the yolk, freed of the vitelline membrane, and transferred to a shallow glass dish containing a small quantity of Locke's solution (after Romeis[7]) placed under a binocular dissecting microscope. After washing off any adhering yolk particles, the blastoderm was carefully spread, dorsal surface uppermost, and the total number of somites noted. The first 4 or 5 somites belonging to the head region were not used in these experiments; hence

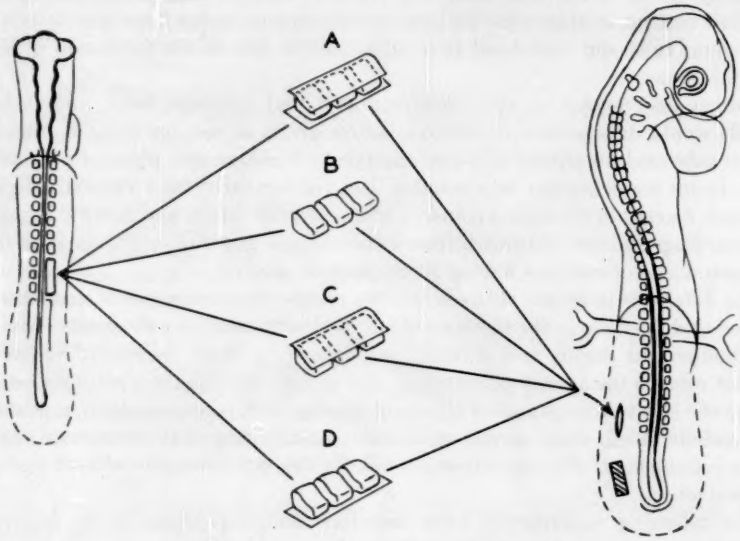


FIG. 1.—Diagram illustrating the 4 types of transplants and their implantation in the embryonic coelom. *A*, somite mesoderm with overlying ectoderm and underlying endoderm; *B*, somite mesoderm alone associated germ layers removed; *C*, somite mesoderm plus overlying ectoderm; *D*, somite mesoderm plus underlying endoderm. Arrow indicates place of insertion of transplant into host coelom.

the earliest stage usable was that of 8 somites. The transplant consisted of a row of somites, usually 3 or 4, excised by means of longitudinal and transverse cuts suitably placed to separate the somites medially from the neural tube and notochord, laterally from the unsegmented lateral plate, and anteriorly and posteriorly from adjacent somites or unsegmented paraxial mesoderm (see Fig. 1).

In the beginning, isolations were made from one side only of each donor. The opposite, unoperated side was fixed as a control and studied in serial section to determine the exact location of the neural crest at the various axial levels of each of the stages tested. Until approximately the 20-somite stage, the neural crest has not begun to migrate from the neural tube in the posteriormost levels. The last 3 or 4 somites were, therefore, always crest-free at the time of isolation from

the neural tube. This was confirmed by the absence of pigment cells, melanocytes, in the grafts from this level when the donors came from a pigmented strain of fowl, the Silver Campine.

Altogether, four different series of experiments were carried out. In series 1, the isolates were transplanted intact, i. e., with ectoderm, mesoderm, and endoderm in their normal relationships (Fig. 1, *A*). In series 2, the somite mesoderm alone was transplanted, both the overlying ectoderm and the underlying endoderm having been removed (Fig. 1, *B*). In series 3, the transplant consisted of mesoderm plus the overlying ectoderm, endoderm only having been removed (Fig. 1, *C*). Finally, in series 4 (Fig. 1, *D*) the mesoderm plus the underlying endoderm was transplanted after removal of the overlying ectoderm. In the last two types of isolations, transplantations were made to the embryonic coelom only. It was found that with a little practice and care the ectoderm and endoderm could be completely dissected from the mesoderm by means of fine tungsten needle-points. This method of removal of the germ layers was, therefore, used throughout the experimentation.

Grafts were removed from the hosts—coelom or chorio-allantoic membrane—8 or 9 days after implantation. They were fixed in Bouin's fluid and stained *in toto* according to the method of Lundvall (see Hamburger[6]) for demonstrating the presence of cartilage. Afterward they were prepared for histological study in serial sections cut at 8 μ and stained with Heidenhain's iron hematoxylin, in order to identify muscle and other structures that might be present and to be certain that the original isolation had been free of neural tube and notochord.

RESULTS FROM INTRACOELOMIC GRAFTING

1. *Transplantation of Somite Mesoderm plus Associated Ectoderm and Endoderm (Table 1).*—This series includes 42 grafts representing a recovery of approximately 40 per cent of the total number of implants made to the embryonic coelom. The grafts were small, rounded, or oval-shaped bodies not exceeding 3 or 4 mm. in greatest dimension. In most cases the outer surface of the graft was covered entirely or in part with skin from which feather germs were developing (Pl. I, Fig. 2). Occasionally skin vesicles with feather germs growing within them were found. The graft was enveloped by host peritoneum. The place of attachment of the graft to the host varied. In 16 cases the attachment was made to the coelomic wall, in 14 cases to the gut or gut mesenteries, and in 12 cases to the Wolffian bodies, ducts, large blood vessels, etc. In relatively few cases was the graft actually imbedded in the coelomic wall. Usually the grafts were quite well vascularized and in the majority of cases were connected with the host by narrow strands of connective tissue containing blood vessels. The age of the donors and the somite levels from which the grafts were obtained may be found in Table 1. It will be noted that grafts were recovered from all stages (8–20 somites) and axial levels tested.

a) Cartilage Formation.—Thirty of the 42 grafts (71 per cent) revealed cartilages of variable size, 1–3 in number and usually in the shape of curved rods, ovals, or spheres (Figs. 2, 8). In the few instances in which the transplant became attached to the host pelvic girdle, the cartilage of the graft tended to become fused with that of the host. As a rule, the cartilages could be easily detected *in toto* by

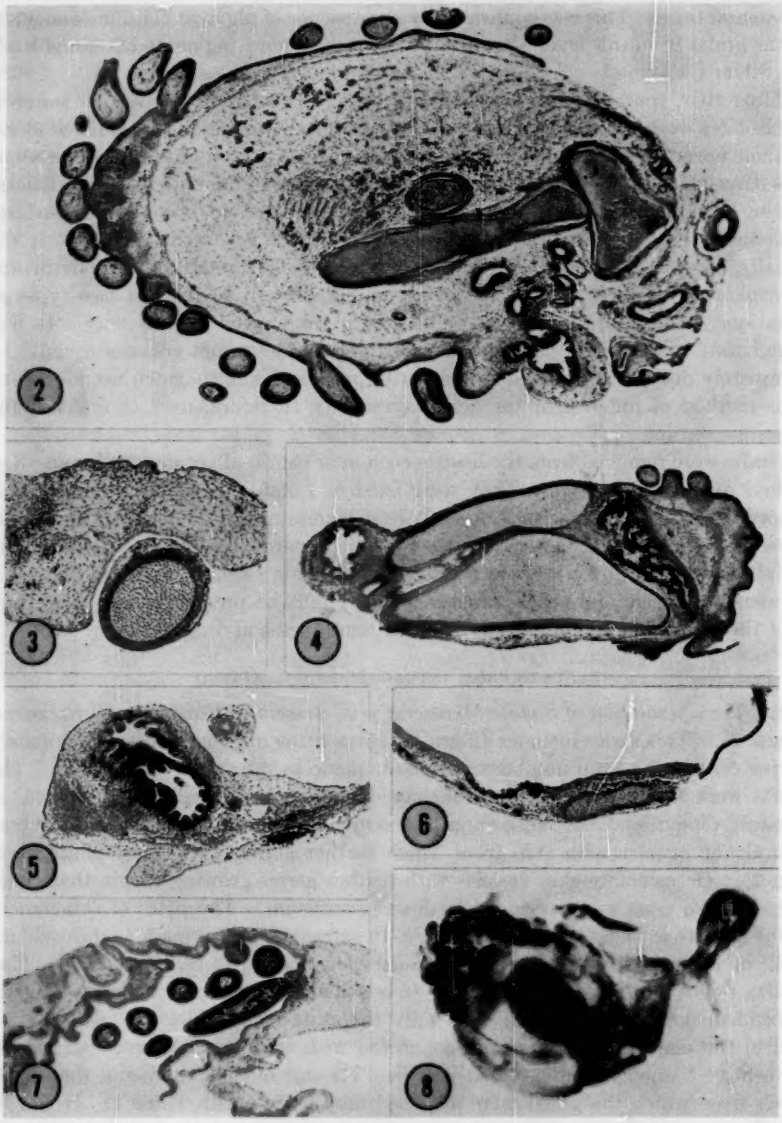


Plate I

INTRA-COELOMIC GRAFTS

FIG. 2.—Section through graft of somite mesoderm plus overlying ectoderm and underlying endoderm from a 9-somite donor. Isolate included somites 7, 8, and 9, from the right side. *Note:* 3 masses of cartilage; skeletal muscle, associated with cartilage, *center of figure;* gut segments, *lower right;* skin with feather germs surrounding most of graft surface. ×30.

FIG. 3.—Section through a mass of cartilage differentiated in graft of somite mesoderm plus overlying ectoderm (endoderm removed) from the last 4 somites of a 10-somite donor. Graft also contained skin and feather germ shown in Fig. 7. ×45.

FIG. 4.—Section through graft of somites 8, 9, and 10, isolated from a 10-somite donor (*right side*). Associated ectoderm and endoderm left in contact with the somite mesoderm. *Note:* 2 large masses of cartilage; bone, *dark mass at right of figure;* gut with smooth muscle coats, *at left of figure;* skin with feather germs surrounding right end of graft. ×30.

FIGS. 5 and 6.—Sections through different regions of the same graft; obtained by isolating somite mesoderm and its associated endoderm (ectoderm removed) from the last 4 somites of a 16-somite donor. Figure 5 shows typical gut with smooth muscle coats. Figure 6 shows mass of cartilage. ×45.

FIG. 7.—Section through skin vesicle with feather germs, differentiated in graft of somite mesoderm and overlying ectoderm (endoderm removed). From the same graft described in Fig. 3. ×30.

FIG. 8.—Intra-coelomic graft of somite mesoderm and its associated ectoderm and endoderm from the last 4 somites of a 12-somite donor. Photograph of cleared whole mount after selective staining for cartilage. The dark, oval-shaped mass in center is cartilage. Feather germs covering surface of graft (*left of figure*) also take up the dye. ×12.

their dark-blue color after staining with Lundvall's technique and clearing in wintergreen oil. Occasionally, however, small bits of cartilage were found in the sectioned material that had not been observed previously in the cleared *in toto* preparations. It was necessary, therefore, that all grafts be sectioned and stained. In the cases listed in Table 1, no trace of neural tube or notochord was found after histological study. Early and advanced stages in the process of chondrogenesis were observed. In 9 cases ossification had begun and in 3 cases was well advanced (Fig. 4).

b) Skeletal Muscle.—This was identified with certainty in 12 out of 42 grafts. It occurred only in the largest grafts and in those in which cartilages were present (Fig. 2). In general, the bundles of fibers were more thinly distributed than in normal control chicks and appeared structurally younger than would be expected

TABLE 1

INTRA-COELOMIC GRAFTS FROM SOMITE MESODERM PLUS ECTODERM PLUS ENDODERM

DONOR AGE		SOMITES GRAFTED	NO. OF GRAFTS RECOVERED	NO. OF GRAFTS WITH CARTILAGE
Total No. of Somites	Stage*			
8	9−	5th–8th	1	1
		6th–8th	2	1
9	9+	7th–9th	3	3
10	10	7th–10th	1	1
		8th–10th	1	1
11	10	8th–11th	3	3
		9th–11th	2	1
		10th–(12)†	1	1
12	10+	6th–8th	1	1
		8th–11th	1	0
		8th–12th	1	0
		9th–12th	4	2
13	11	10th–12th	1	0
		10th–13th	2	0
		11th–13th	1	1
14	11	7th–9th	2	2
		11th–14th	2	2
		13th–(15)†	1	1
15	11+	12th–15th	5	3
16	12	13th–16th	1	1
17	12	14th–17th	1	1
18	12+	15th–18th	1	1
19	13	14th–16th	1	1
		17th–19th	1	1
20	13	15th–17th	1	0
		17th–20th	1	1

* Staged according to Hamburger-Hamilton series.

† Inclusion of adjacent unsegmented portion of paraxial plate equivalent to 1 somite in dimension.

in view of the total age of the grafted tissues (10–11 days). The criterion for the identification of skeletal muscle was the appearance of striations among at least some of the muscle bundles in each of the 12 cases. In 5 other cases long multinucleate fibers resembling those of skeletal muscle, prior to the formation of striations, were observed but failed to show the characteristic striations.

c) Other Structures.—Skin and feather germs, as already mentioned, were conspicuously present in the great majority of the grafts (35 out of 42 cases). Segments of gut with smooth muscle coats were also of frequent occurrence (37 out of 42 cases). The occasional presence of mesonephric tubules (7 cases) may be correlated with the inclusion of some of the mesoderm cells of the intermediate cell mass in the original isolate.

2. *Transplantation of Somite Mesoderm Alone.*—This series includes 16 cases in which a row of 3 or 4 somites was excised after the ectoderm and endoderm covering the area of excision had been carefully stripped from the donor blastoderms (8–20 somite stages). The isolated mesoderm was transplanted in the usual way to the coelom of host embryos. When the hosts were dissected and examined 8 days later. no graft or recognizable growth of any kind was found. These negative results are in sharp contrast to the high percentage of positive results obtained from the preceding series in which the ectoderm and endoderm were not removed from the mesoderm before transplantation.

Thus, having demonstrated that somitic mesoderm alone does not undergo differentiation, whereas the same mesoderm left in normal contact with ectoderm and endoderm does undergo differentiation in a high percentage of cases, the question arises as to whether both ectoderm and endoderm are necessary constituents. Would somite mesoderm in contact with either one or the other of these germ layers react to form cartilage and skeletal muscle? To answer this question the following two series of experiments were carried out.

TABLE 2

INTRA-COELOMIC GRAFTS FROM SOMITE MESODERM PLUS ECTODERM

DONOR AGE		SOMITES GRAFTED	NO. OF GRAFTS RECOVERED	NO. OF GRAFTS WITH CARTILAGE
Total No. of Somites	Stage*			
11	10	6th–11th	1	1
14	11	9th–13th	1	0
15	11+	6th–9th	1	1
		9th–14th	2	1
		11th–15th	2	1
16	12	9th–15th	1	0
17	12	12th–16th	2	2
18	12+	11th–16th	2	1
20	13	10th–14th	1	0
		15th–19th	1	1

* Staged according to Hamburger-Hamilton series.

3. *Transplantation of Somite Mesoderm plus Overlying Ectoderm (Table 2).*—This series includes 14 grafts. The donors ranged in age from 11 to 20 somites. On the whole, these grafts were somewhat smaller than those obtained from series 1, in which all three germ layers were included; otherwise they were quite similar in general appearance, attachment, etc. Cartilages differentiated from the mesoderm in 8 of the 14 cases; in 2 of these, ossification had begun. In 3 of the larger grafts containing cartilage, small groups of fibers closely resembling skeletal muscle

TABLE 3

INTRA-COELOMIC GRAFTS FROM SOMITE MESODERM PLUS ENDODERM

Donor Age		Somites Grafted	No. of Grafts Recovered	No. of Grafts with Cartilage
Total No. of Somites	Stage*			
8	9−	4th–7th	1	1
13	11	7th–12th	1	0
14	11	9th–13th	2	0
16	12	9th–13th	1	1
17	12	9th–12th	1	0
		12th–16th	2	0
18	12+	13th–17th	2	0
19	13	7th–12th	2	0
		12th–16th	1	0
21	13+	14th–19th	1	0

* Staged according to Hamburger-Hamilton series.

were observed; but, since striations were not detected, the identification of skeletal muscle could not be made with certainty. Skin and feather germs were common and showed normal differentiation (Fig. 7). A few epithelial tubules resembling those of the nesonephros occurred now and then, but glomeruli were not found.

4. *Transplantation of Somite Mesoderm plus Underlying Endoderm (Table 3).*— The 14 grafts obtained in this series were relatively insignificant in appearance, and most of them could have been easily overlooked without scrutiny. Small lumps of cartilage were found in 2 cases only—one from an 8-somite donor, the other from a 16-somite donor (Fig. 6). The majority of the grafts contained small segments of gut with associated smooth muscle (Fig. 5). No tissue bearing any resemblance to skeletal muscle was observed.

TABLE 4

CHORIO-ALLANTOIC GRAFTS FROM SOMITE MESODERM PLUS ECTODERM AND ENDODERM

Donor Age		Somites Grafted	No. of Grafts Recovered	No. of Grafts with Cartilage
Total No. of Somites	Stage*			
10	10	7th–10th	10	2
12	11−	9th–12th	6	0
13	11	6th–9th	2	0
		10th–13th	8	2
14	11+	11th–14th	3	1
		10th–14th	1	0
15	12−	5th–7th	1	1
		8th–11th	2	0
		12th–15th	7	4
16	12	7th–11th	2	1
		12th–15th	2	1
		12th–16th	1	0
		13th–16th	1	1
23	14+	20th–23d	2	1
		21st–23d	1	0
24	15−	13th–15th	1	1
		17th–20th	1	1
25	15	22nd–25th	2	1
26	15+	23rd–26th	1	1
27	16	20th–23d	1	1
		24th–27th	1	1
28	16	25th–28th	2	1
30	17−	10th–14th	1	0
		16th–20th	1	1
		21th–25th	1	1
		26th–30th	1	0

* Staged according to Hamburger-Hamilton series.

RESULTS FROM CHORIO-ALLANTOIC GRAFTING

1. Transplantation of Somite Mesoderm plus Overlying Ectoderm and Underlying Endoderm (*Table 4*).—The results of this series are in good agreement with those obtained from grafting the whole tissue complex to the embryonic coelom. The principal difference between the two series lies in the percentage of grafts showing good growth and differentiation of the various tissue derivatives of the three germ layers included. The embryonic coelom unquestionably provides a more normal environment for growth and differentiation of the transplant. This is especially noticeable in the earlier somite stages tested (compare Tables 1 and 4). Perceptible thickenings were found on the chorio-allantoic membrane at the site of transplantation in 46 cases from donors ranging in age from 10 to 16 somites. Although these were classified as "grafts" at the time of removal, upon histological examination it was found that cartilage, muscle, gut, skin, and feather germs had differentiated in a little less than a third of the total number. In general, the size of a graft is a rather good indication of the degree of differentiation attained. This applies also to intra-coelomic grafts. Transplants that actually underwent differentiation on the chorio-allantois were essentially similar to those developing in the coelom, although they were nearly always smaller in size.

a) Cartilage Formation.—Thirteen of the 46 grafts (28 per cent) from donors ranging in age from 10 to 16 somites developed cartilages, 1–3 in number, in the shape of curved rods, ovals, or spheres. In 2 of these cases ossification had begun. Whereas the percentage of cartilage differentiation was considerably less than that obtained from intra-coelomic grafts of the same donor age, in which cartilage formed in 13 out of 22 cases (59 per cent), the differentiation was qualitatively the same.

b) Other Structures.—No striated skeletal muscle fibers were found in any of the grafts. In two cases, scattered bundles of fibers associated with cartilage and closely resembling young skeletal muscle were observed. The most frequently occurring structures were gut segments surrounded by smooth muscle, skin, and feather germs. Epithelial tubules occurred in the majority of the grafts, many of which were too nondescript to warrant identification. This was especially true for those grafts displaying feeble growth. The presence of mesonephric tubules could again be correlated with the original inclusion of nephrogenous cells from the intermediate cell mass.

Since the percentage of grafts showing skeletogenous tissue was low in the above-described grafts from 12- to 16-somite donors, this series of experiments was extended to include slightly older donors of from 23 to 30 somites (see Table 4). It is to be noted especially that cartilages now occurred in 10 out of 16 grafts recovered, or 62.5 per cent—a substantial increase of 34.5 per cent. Although bundles of muscle fibers undoubtedly skeletal in character were found in 4 cases, again no striations appeared. Gut, smooth muscle, skin, and feather germs were relatively common, as in the above series.

2. Transplantation of Somite Mesoderm Alone.—In only 3 cases out of 33, in which somite mesoderm from 10–20-somite donors was freed of its associated ectoderm and endoderm and transplanted to the chorio-allantoic membrane, was any thickening found at the transplantation site. These 3 cases contained no cartilage

or any other recognizable tissue but were composed entirely of mesenchyme invaded by blood vessels from the chorio-allantoic membrane.

SUMMARY AND CONCLUSIONS

The results described demonstrate clearly that somite mesoderm of the trunk region of early embryos (8–20 somites) is capable of differentiating into skeletal tissue without the presence of neural tube and notochord. Such differentiation, however, can be achieved only when the somite mesoderm is transplanted in contact with either the overlying ectoderm or the underlying endoderm or both. Maximal differentiation of both cartilage and skeletal muscle was obtained when the two associated germ layers were present. Removal of the endoderm affected growth and differentiation to a lesser degree than did removal of ectoderm; mesoderm plus endoderm, for instance, produced only a minimal amount of cartilage and no trace of skeletal muscle. Mesoderm alone failed to differentiate. Thus it would appear that if the normal spatial tissue relationships are not entirely disrupted, early somite mesoderm can realize its inherent developmental potentialities established in presomite stages (for pertinent information concerning the localization of the somite mesoderm in presomite blastoderms, see Rudnick[8]). These results are in line with those of Muehmore,[9] in which it was found that muscle differentiation in amphibian embryos is dependent upon the maintenance of a certain proportion of the normal relationships between the somite and its surrounding tissues.

The fact that early somite mesoderm left in contact with its associated germ layers can differentiate into skeletal tissue in the absence of the neural tube, when grafted to a favorable environment, does not imply that this structure normally has no effect on the developing somite. The neural tube is an integral part of the somite environment and as such (if for no other reason) would be expected to contribute importantly. Actually, it was demonstrated 25 years ago, by Murray and Selby,[4] that the frequency of differentiation of cartilage, bone, and skeletal muscle in chorio-allantoic grafts of somite mesoderm plus ectoderm and endoderm, was enhanced considerably by the inclusion of the adjacent neural tube. During the course of the present investigation it was also found that whenever a segment of the neural tube was either purposely or accidentally included in the original isolate, the development of cartilage and skeletal muscle was definitely intensified. There is no doubt, then, that the presence of neutral tissue (spinal cord) somehow steps up the growth and differentiation of both cartilage and skeletal muscle. Whether the influence of the neural tube is specifically one of induction in the early somite stages, as postulated in recent years, not only for the chick[1–3] but for other vertebrate embryos,[10–12] is indeed questionable in the light of the present experimental results. Whatever the "influence" of the neural tube on early somite mesoderm may be, the present grafting experiments have shown quite clearly that it can be compensated for, to a considerable extent, by the presence of both associated germ layers (see also Hoadley[13]).

There is no evidence that the neural crest affects the formation of cartilage in grafts. Cartilages formed equally as well in isolates removed before the neural crest had begun its migration from the neural tube as in those removed after migration had begun. It would appear that in the chick, as in other vertebrate embryos,

the trunk neural crest is used up primarily in the formtion of structures other than cartilage (see Hörstadius[14]).

As regards the failure of early somite mesoderm to differentiate when completely isolated, the results of this investigation are in agreement with those of other workers. It is not unreasonable to assume that the failure to differentiate is related to the consistency of the mesenchymal tissue. Early mesenchyme is notably soft and spongy and exhibits a marked tendency to spread when isolated. The fact that differentiation does take place when some of its adjacent tissues are included in the graft may simply mean that they lessen or prevent spreading to the extent, at least, of maintaining the critical mass necessary for differentiation (cf. Muchmore[15]). By the third or fourth days of incubation the somite mesoderm has become sufficiently solid and compact to prevent loss of mass by spreading and can therefore differentiate without further tissue support when isolated.

While adjacent tissues may play a role in holding early mesoderm together and thus provide loci for differentiation, the "support" of adjacent tissues should not be interpreted as merely of a mechanical nature. Rather, it is to be thought of as a physiological interaction between the mesodermal cells and those of neighboring tissues.

In view of the emphasis that has been placed recently upon the inductive influence of the neural tube (and notochord) on the differentiation of early somite mesoderm into skeletal tissue, the present study may serve well to direct attention to other neighboring tissues, such as the ectoderm and the endoderm, which clearly, also, play an important part in somite development.

* These experiments were begun at the Hubrecht Laboratory, Utrecht, Holland, in 1956, as a part of the project of the second international research team in experimental embryology directed by Drs. Pieter D. Nieuwkoop and Mary E. Rawles. The work was continued the following year at Johns Hopkins University by one of us (T. S.) under the supervision of Dr. Rawles and was supported in part by a grant from the National Institutes of Health. The authors are indebted to Mr. John S. Spurbeck, of the Johns Hopkins University, for the preparation of the illustrations.

[1] R. L. Watterson, I. Fowler, and B. J. Fowler, *Am. J. Anat.*, **95**, 337, 1954.

[2] G. Avery, M. Chow, and H. Holtzer, *J. Exptl. Zool.*, **132**, 409, 1956.

[3] J. W. Lash and S. W. Holtzer, *Anat. Rec.*, **127**, 323, 1957.

[4] P. D. F. Murray and D. S. Selby, *J. Anat.*, **67**, 563, 1933.

[5] W. L. Straus, Jr., and M. E. Rawles, *Am. J. Anat.*, **92**, 471, 1953.

[6] V. Hamburger, *A Manual of Experimental Embryology* (Chicago: University of Chicago Press, 1942).

[7] B. Romeis, *Taschenbuch der mikroskopischen Technik* (Munich and Berlin: R. Oldenbourg, 1924), p. 18.

[8] D. Rudnick, in *Analysis of Development*, ed. B. H. Willier, P. A. Weiss, and V. Hamburger (Philadelphia and London: W. B. Saunders Co., 1955), p. 297.

[9] W. B. Muchmore, *J. Exptl. Zool.*, **118**, 137, 1951.

[10] H. Holtzer and S. R. Detwiler, *J. Exptl. Zool.*, **123**, 335, 1953.

[11] C. Grobstein and G. Parker, *Proc. Soc. Exptl. Biol. Med.*, **85**, 477, 1954.

[12] C. Grobstein and H. Holtzer, *J. Exptl. Zool.*, **128**, 333, 1955.

[13] L. Hoadley, *J. Exptl. Zool.*, **42**, 143, 1925.

[14] S. Hörstadius, *The Neural Crest* (London, New York, and Toronto: Oxford University Press, 1950).

[15] W. B. Muchmore, *J. Exptl. Zool.*, **134**, 293, 1957.

ERRATA: DIFFERENTIABLE MAPPINGS IN THE SCHOENFLIES PROBLEM

BY MARSTON MORSE

In the article of the above title appearing in these PROCEEDINGS, **44,** pp. 1068–1072, 1958, the following corrections and additions should be made:

Page 1069

In the last line of text matter, N_q should be $\mathfrak{N}_q$

Page 1070

In line 8, the first of three signs $\cup$ should be $\cap$.
In the last line, suppose $K - G$ and $K - B$ are arc-wise connected.

Page 1072

In line 10, replace P by $P \cup \mathring{K}$.
In lines 11, 12, and 13, delete references to D_1.

INDEX

A_2 erythrocytes in A_1 blood (Atwood), 1054

Aberrations, chromosomal, in pre-spermatocytic cells of irradiated male mice (Griffen), 691

Acetate, a calcium-sparing factor in nitrogen fixation (Esposito and Wilson), 472

Acids, fatty, enzymatic synthesis of, by aldol condensation (Brady), 993

ACS, GEORGE. *See* Weiss, Samuel B., 189

———. *See* Zachau, Hans Georg, 885

Actinomycins, nomenclature of (Waksman, Katz, and Vining), 602

AD-4 locus in *N. crassa*, complementation at (Woodward, Partridge, and Giles), 1237

ADAMS, JANE N., and LURIA, S. E. Transduction by Bacteriophage P1: Abnormal Phage Function of the Transducing Particles, 590

Adenosine amino acid esters, isolation of, from a ribonuclease digest of liver ribonucleic acid (Zachau, Acs, and Lipmann), 885

Adenyl tryptophan, enzymatic formation of (Kingdon, Webster, and Davie), 757

ADLER, JULIUS, LEHMAN, I. R., BESSMAN, MAURICE J., SIMMS, E. S., and KORNBERG, ARTHUR. Enzymatic Synthesis of Deoxyribonucleic Acid. IV. Linkage of Single Deoxynucleotides to the Deoxynucleoside Ends of Deoxyribonucleic Acid, 641

ADLER, JULIUS. *See* Bessman, Maurice J., 633

———. *See* Lehman, I. R., 1191

Aeronomy, chemical (Kaplan and Barth), 105

Aldolase, mechanism of action (Rose), 10

ALEXANDER, MARY L. Biological Damage in Developing Germ Cells of *Drosophila virilis* in Oxygen and Nitrogen with 14-Mev Neutrons, 1217

ALLEN, ESTHER. *See* Schweet, Richard S., 173, 1029

ALLFREY, V. G., and MIRSKY, A. E. Some Effects of Substituting the Deoxyribonucleic Acid of Isolated Nuclei with Other Polyelectrolytes, 981

Amides, mode of protonation of (Fraenkel and Niemann), 688

Amino acid: incorporation in pigeon pancreas fractions (Weiss, Acs, and Lipmann), 189; incorporation into ribonucleic acid (Schweet, Bovard, Allen, and Glassman), 173; incorporation with cell-free *Tetrahymena* preparations (Mager and Lipmann), 305

ANDERSON, I. C. *See* Fuller, R. C., 239, 518

ANKER, H. S. *See* Catravas, G. N., 1097

ANSELL, NORMA J. *See* Springer, Georg F., 182

Approximation, successive, dynamic programming, and monotone convergence (Bellman), 578

Arithmetical function, Von Sterneck, and restricted partitions with respect to a modulus, supplement to (Nicol and Vandiver), 917

ARNOLD, WILLIAM, STEELE, RICHARD, and MUELLER, HELMUT. On the Magnetic Asymmetry of Muscle Fibers, 1

ATWOOD, K. C. Presence of A_2 Erythrocytes in A_1 Blood, 1054

AUSTIN, D. G. New Proof of the Strong Markov Theorem of Chung, A, 575

Autoradiography with tritiated thymidine to reveal cellular proliferation in the mouse (Hughes, Bond, Brecher, Cronkite, Painter, Quastler, and Sherman), 476

Bacteria, modification of ultraviolet-induced mutation and survival in (Doudney and Haas), 390

Bacterial system, cell-free, causing protein biosynthesis (Beljanski and Ochoa), 494, 1157

Bacterial transformation data, interpretation of (Cavalieri and Rosenberg), 853

Bacteriophage P1, transduction by (Adams and Luria), 590

BAER, HANS HELMUT, and FISCHER, HERMANN O. L. New Way for the Synthesis of 3-Amino Sugars, A, 991

BARKER, H. A., WEISSBACH, H., and SMYTH, R. D. Coenzyme Containing Pseudovitamin B_{12}, A, 1093

BARNER, HAZEL D. *See* Cohen, Seymour S., 1004

Barriers, potential, to internal rotation in molecules (Pauling), 211

BARRON, D. H. *See* Meschia, G., 483

BARTH, CHARLES A. *See* Kaplan, Joseph, 105

BASSHAM, J. A., KIRK, M., and CALVIN, M. Effects of Hydroxylamine on the $C^{14}O_2$ Fixation Pattern during Photosynthesis, The, 491

BAXTER, MARGERY G. *See* Raper, John R., 889

BELJANSKI, MIRKO, and OCHOA, SEVERO. Protein Biosynthesis by a Cell-free Bacterial System, 494

———. Protein Biosynthesis by a Cell-free Bacterial System. II. Further Studies on the Amino Acid Incorporation Enzyme, 1157

BELLMAN, RICHARD. Dynamic Programming, Successive Approximation, and Monotone Convergence, 578

BELLMAN, RICHARD, and KALABA, ROBERT. Invariant Imbedding, Wave Propagation, and the WKB Approximation, 317

BELLMAN, RICHARD, and LEHMAN, SHERMAN. Functional Equations in the Theory of Dynamic Programming. IX. Variational Analysis, Analytic Continuation, and Imbedding of Operators, 905

Bending, minimum, of orbitals (Eyring, Stewart, and Smith), 259

BENESCH, REINHOLD, and BENESCH, RUTH E. Thiolation of Proteins, 848

BENESCH, RUTH E. *See* Benesch, Reinhold, 848

BENNETT, M. F. *See* Brown, Frank A., Jr., 290

BENZER, SEYMOUR, and FREESE, ERNST. Induction of Specific Mutations with 5-Bromouracil, 112

BERG, P., and OFENGAND, E. J. Enzymatic Mechanism for Linking Amino Acids to RNA, An, 78

BERKOWITZ, JOAN B. *See* Fuoss, Raymond M., 27
Bertini's theorem for local domains (Chow), 580
BESSMAN, MAURICE J., LEHMAN, I. R., ADLER, JULIUS, ZIMMERMAN, STEVEN B., SIMMS, E. S., and KORNBERG, ARTHUR. Enzymatic Synthesis of Deoxyribonucleic Acid. III. The Incorporation of Pyrimidine and Purine Analogues into Deoxyribonucleic Acid, 633
BESSMAN, MAURICE J. *See* Adler, Julius, 641
———. *See* Lehman, I. R., 1191
BHARUCHA-REID, A. T., and RUBIN, HERMAN. Generating Functions and the Semigroup Theory of Branching Markov Processes, 1057
Biosynthesis: of fatty acid, regulated by lipogenin (Catravas and Anker), 1097; of isoleucine and valine in *N. crassa* (Wagner, Radhakrishnan, and Snell), 1047; protein, by cell-free bacterial system (Beljanski and Ochoa), 494, 1157
BISHOP, ERRETT. Measures Orthogonal to Polynomials, 278
BISHOP, NORMAN I. Vitamin K, an Essential Factor for the Photochemical Activity of Isolated Chloroplasts, 501
BLACK, L. M. Wound-Tumor, 363
BLOCH, K. *See* Chaykin, S., 998
BLOCH, KONRAD. *See* Rilling, H., 167
Blood characteristics common to alcoholic males (Williams, Pelton, Hakkinen, and Rogers), 216
BOND, V. P. *See* Hughes, W. L., 476
Bond orbitals, nature of (Pauling), 211
BOONE, WILLIAM W. Word Problem, The, 1061
BOREK, ERNEST, PONTICORVO, LAURA, and RITTENBERG, D. Protein Turnover in Micro-organisms, 369
BOREK, ERNEST, and RYAN, ANN. Transfer of Irradiation-elicited Induction in a Lysogenic Organism, The, 374
BOURNE, SAMUEL, and ZASSENHAUS, HANS. On the Semiradical of a Semiring, 907
BOVARD, FREEMAN C. *See* Schweet, Richard S., 173
BOYER, P. D., and STULBERG, M. P. Tracing of the in Vivo Path from Amino Acid to Protein, 92
BRADY, ROSCOE O. Enzymatic Synthesis of Fatty Acids by Aldol Condensation, The, 993
BRAUN, ARMIN C. Physiological Basis for Autonomous Growth of the Crown-Gall Tumor Cell, A, 344
BRAUN, WERNER. *See* Firshein, William, 918
BRECHER, G. *See* Hughes, W. L., 476
BROWN, DAVID H. *See* Illingworth, Barbara, 1180
BROWN, FRANK A., JR., BENNETT, M. F., and WEBB, H. M. Monthly Cycles in an Organism in Constant Conditions during 1956 and 1957, 290
BRUCE, VICTOR. *See* Pittendregh, Colin, 965
BÜYÜKÖZER, ILHAN. *See* Seno, Tamikazu, 1274

CAGLE, F. W., JR. *See* Eyring, Henry, 120
———. *See* Manson, James E., 156
Calculus, operational, with differentiable functions (Nachbin), 698
CALVIN, M. *See* Bassham, J. A., 491
———. *See* Metzner, Helmut, 205
———. *See* Moses, V., 260
———. *See* Tollin, G., 1035
Carbohydrates, crystalline, paramagnetic resonance study of irradiation damage in (Williams, Geusic, Wolfrom, and McCabe), 1127
Carbon, path of, in photosynthesis (Moses and Calvin), 260
Carbon dioxide, thermal conductivity of, in the region of the critical point (Guildner), 1149
Carboxy-ketopentitol diphosphates as products of photosynthesis (Moses and Calvin), 260
CO_2 fixation products in photosynthesis (Metzner, Metzner, and Calvin), 205
CARSON, HAMPTON L. Increase in Fitness in Experimental Populations Resulting from Heterosis, 1136
Cartilage formation in somite grafts of chick blastoderm (Seno and Büyüközer), 1274
CASE, MARY E., and GILES, NORMAN H. Evidence from Tetrad Analysis for Both Normal and Aberrant Recombination between Allelic Mutants in *Neurospora crassa*, 378
CATRAVAS, G. N., and ANKER, H. S. On the Regulation of Fatty Acid Biosynthesis by Lipogenin, 1097
CAVALIERI, LIEBE F., and ROSENBERG, BARBARA H. Interpretation of Bacterial Transformation Data, The, 853
Cell, initiator, for slime mold aggregation (Ennis and Sussman), 401
Cell-migration patterns, modification of (DeHaan), 32
Cellular proliferation in the mouse (Hughes, Bond, Brecher, Cronkite, Painter, Quastler and Sherman), 476
CESARI, L., and TURNER, L. H. On the Concept of Surface Integral, 42
CHANDRASEKHAR, S. On the Diffuse Reflection of a Pencil of Radiation by a Plane-parallel Atmosphere, 933
———. On the Equilibrium Configuration of an Incompressible Fluid with Axisymmetric Motions and Magnetic Fields, 842
CHANDRASEKHAR, S., and WOLTJER, L. On Force-free Magnetic Fields, 285
CHARNES, A., and COOPER, W. W. Extremal Principles for Simulating Traffic Flow in a Network, 201
———. Strong Minkowski-Farkas-Weyl Theorem for Vector Spaces over Ordered Fields, The, 914
CHAYKIN, S., LAW, J., PHILLIPS, A. H., TCHEN, T. T., and BLOCH, K. Phosphorylated Intermediates in the Synthesis of Squalene, 998
CHEADLE, VERNON I. *See* Esau, Katherine, 546
CHOVNICK, ARTHUR. Aberrant Segregation and Pseudoallelism at the Garnet Locus in *Drosophila melanogaster*, 333
CHOW, WEI-LIANG. On the Theorem of Bertini for Local Domains, 580

CHOW, WEI-LIANG, and IGUSA, JUN-ICHI. Cohomology Theory of Varieties over Rings, 1244
CHRISTENSEN, CARL J. *See* Eyring, Henry, 120
Chromosomal polymorphism in *Drosophila willistoni* (Townsend), 38
Chromosomes of man, somatic (Tjio and Puck), 1229
Chung's Strong Markov theorem, new proof of (Austin), 575
CHURCH, BROOKS. *See* Halvorson, Harlyn, 1171
CLARKE, JOHN V., JR. *See* Rodebush, Worth H., 536
Cochlear potentials in the cat in response to high-frequency sounds (Wever, Vernon Rahm, and Strother), 1087
Coenzymatic function of estradiol-17-β (Talalay, Hurlock, and Williams-Ashman), 862
Coenzyme containing pseudovitamin B_{12} (Barker, Weissbach, and Smyth), 1093
COHEN, SEYMOUR S., FLAKS, JOEL G., BARNER, HAZEL D., LOEB, MARILYN R., and LICHTENSTEIN, JANET. Mode of Action of 5-Fluorouracil and Its Derivatives, The, 1004
Cohomology theory of varieties over rings (Chow and Igusa), 1244
COLBERT, EDWIN H. Tetrapod Extinctions at the End of the Triassic Period, 973
Collagen in the frog embryo (Edds), 296
Collagen macromolecules: sonic fragmentation of (Nishihara and Doty), 411; sonically fragmented, interaction properties of Hodge and Schmitt), 418
COMMONER, BARRY, and LIPPINCOTT, BARBARA B. Light-induced Free Radicals in FMN and Flavoprotein Enzymes, 1110
COMMONER, BARRY, LIPPINCOTT, BARBARA B., and PASSONNEAU, JANET V. Electron-Spin Resonance Studies of Free-Radical Intermediates in Oxidation-Reduction Enzyme Systems, 1099
COMMONER, BARRY, SHEARER, GEORGIA B., and STRODE, CLARICE. Linear Analysis of Tobacco Mosaic Virus, 1117
Complementation at AD-4 locus in *N. crassa* (Woodward, Partridge, and Giles), 1237
Computing machine, rapid, an instrument in discovery of new relations in theory of numbers (Vandiver), 459
Conductivity, thermal, of carbon dioxide in region of critical point (Guildner), 1149
CONGDON, C. C. *See* Gengozian, N., 560
Control systems, epigenetic (Nanney), 712
Convergence, monotone, dynamic programming and successive approximation (Bellman), 578
COOPER, W. W. *See* Charnes, A., 201, 914
Copoly-L-Lysine-L-Glutamic Acid, solution properties and configurations of (Doty, Imahori, and Klemperer), 424
CORI, CARL F. *See* Illingworth, Barbara, 1180
COURNAND, A. *See* Fritts, H. W., Jr., 1079
CRAWFORD, IRVING P., and YANOFSKY, CHARLES. On the Separation of the Tryptophan Synthetase of *Escherichia coli* into Two Protein Components, 1161
CRONKITE, E. P. *See* Hughes, W. L., 476
Crystal structure and information theory (McLachlan), 948
Cycles, monthly, in an organism in constant conditions (Brown, Bennett, and Webb), 290

DAVIE, EARL W. *See* Kingdon, Henry S., 757
DEHAAN, ROBERT L. Modification of Cell-Migration Patterns in the Early Chick Embryo, 32
Dehydrogenases, coenzyme binding to (Shifrin and Kaplan), 177
DNA: effect of molecular weight of, on pneumococcal transformation (Litt, Marmur, Ephrussi-Taylor, and Doty), 144; enzymatic synthesis of (Bessman, Lehman, Adler, Zimmerman, Simms, and Kornberg), 633; enzymatically synthesized, chemical composition of (Lehman, Zimmerman, Adler, Bessman, Simms, and Kornberg), 1191; of isolated nuclei, some effects of substituting with other polyelectrolytes (Allfrey and Mirsky), 981; property of sonic fragments of (Doty, McGill, and Rice), 432; replication of, in *E. coli* (Meselson and Stahl), 671
DNA digest, selective effects of, upon pneumococci of different virulence (Firshein and Braun), 918
Deoxyribonucleate, transformation of biochemically deficient strains of *B. subtilis* by (Spizizen), 1072
Dibasic acids, ionization of individual groups in (Edsall, Martin, and Hollingworth), 505
DIMOND, A. E. *See* Husain, Akhtar, 594
Dipicolinic acid regulating electron transport (Halvorson, Doi, and Church), 1171
Distributions, foundations of theory of (Gál), 1248
DOBZHANSKY, THEODOSIUS, and PAVLOVSKY, OLGA. Interracial Hybridization and Breakdown of Coadapted Gene Complexes in *Drosophila willistoni*, 622
DOI, ROY. *See* Halvorson, Harlyn, 1171
DOLD, ALBRECHT, and PUPPE, DIETER. Non-additive Functors, Their Derived Functors, and the Suspension Homomorphism, 1065
Domains, local, Bertini's theorem for (Chow), 580
DOTY, P., IMAHORI, K., and KLEMPERER, E. Solution Properties and Configurations of a Polyampholytic Polypeptide: Copoly-L-Lysine-L-Glutamic Acid, The, 424
DOTY, PAUL, MCGILL, BARBARA BUNCE, and RICE, STUART A. Properties of Sonic Fragments of Deoxyribose Nucleic Acid, The, 432
DOTY, PAUL. *See* Litt, Michael, 144
———. *See* Nishihara, Tomio, 411
Doubling, *l*-type, in NH_2 (Garing and Nielsen), 467
DOUDNEY, C. O., and HAAS, F. L. Modification of Ultraviolet-induced Mutation Frequency and Survival in Bacteria by Post-Irradiation Treatment, 390

DOWLING, JOHN E., and WALD, GEORGE. Vitamin A Deficiency and Night Blindness, 648
Drosophila, genetic studies of irradiated natural populations of (Stone and Wilson), 565
Dynamic programming, functional equations in theory of (Bellman and Lehman), 905

EDDS, MAC V., JR. Development of Collagen in the Frog Embryo, 296
EDSALL, JOHN T., MARTIN, R. BRUCE, and HOLLINGWORTH, BARBARA K. Ionization of Individual Groups in Dibasic Acids, with Application to the Amino and Hydroxyl Groups of Tyrosine, 505
EISENHART, LUTHER P. Spaces for which the Ricci Scalar R is Equal to Zero, 695
Electric potential differences across goat placentas (Meschia, Wolkoff, and Barron), 483
Electrohysterogram, human, wave forms and implications of (Larks), 820
Electron-donor and -acceptor properties of purines, pyrimidines, pteridines, flavins, and aromatic amino acids (Pullman and Pullman), 1197
Electron-spin resonance studies of free-radical intermediates in oxidation-reduction enzyme systems (Commoner, Lippincott, and Passonneau), 1099
Electron transport regulated by dipicolinic acid (Halvorson, Doi, and Church), 1171
Elements, sieve, wall thickening in (Esau and Cheadle), 546
EMERSON, MARY R. *See* Emerson, Sterling, 668
EMERSON, STERLING, and EMERSON, MARY R. Production, Reproduction, and Reversion of Protoplast-like Structures in the Osmotic Strain of *Neurospora crassa*, 668
Endospores, bacterial, dormancy of (Halvorson, Doi, and Church), 1171
ENNIS, HERBERT L., and SUSSMAN, MAURICE. Initiator Cell for Slime Mold Aggregation, The, 401
Enzymatic synthesis of deoxyribonucleic acid (Bessman, Lehman, Adler, Zimmerman, Simms, and Kornberg), 633, 1191
Enzyme, receptor-destroying, effect on human erythrocyte agglutinogens (Springer and Ansell), 182
Enzyme which stimulates incorporation of amino acid (Beljanski and Ochoa), 1157
Enzyme systems, oxidation-reduction, electron-spin resonance studies of free-radical intermediates in (Commoner, Lippincott, and Passonneau), 1099
Enzymes: extracellular, of Dutch elm disease pathogen, function of (Husain and Dimond), 594; FMN and flavoprotein, light-induced free radicals in (Commoner and Lippincott), 1110
Enzymic activity of subtilisin-modified ribonuclease (Richards), 162
EPHRUSSI-TAYLOR, HARRIETT. *See* Litt, Michael, 144
Equations: diffusion, class of (Geeslin), 788; functional, in theory of dynamic programming (Bellman and Lehman), 905; integral, from elastostatics (Fredricks), 309
Equilibrium configurations of incompressible fluid with axisymmetric motions and magnetic fields (Chandrasekhar), 842
Equilibrium, hydromagnetic (Woltjer), 833
Erythrocyte agglutinogens M and N, inactivated by influenza viruses and receptor destroying enzyme (Springer and Ansell), 182
Erythrocytes, age effect on enzyme activity of (Marks, Johnson, and Hirschberg), 529
ESAU, KATHERINE, and CHEADLE, VERNON I. Wall Thickening in Sieve Elements, 546
ESPOSITO, RAYMOND G., and WILSON, P. W. Acetate as a Calcium-sparing Factor in Nitrogen Fixation by *Azotobacter vinelandii*, 472
Estradiol-17-β, coenzymatic function of (Talalay, Hurlock, and Williams-Ashman), 862
EVANS, G. C. Surface of Given Space-Curve Boundary, 786
EWING, MAURICE. *See* Oliver, Jack, 780
Excitation energy, resonance transfer of, between amino acids in proteins (Karreman, Steele, and Szent-Györgyi), 140
EYRING, HENRY, CAGLE, F. W., JR., and CHRISTENSEN, CARL J. Theory of Optimum Conditions for Phase Transitions, A, 120
EYRING, HENRY, REE, TAIKYUE, and HIRAI, NISHIO. Significant Structures in the Liquid State, 683
———. Viscosity of High Polymers—the Random Walk of a Group of Connected Segments, The, 1213
EYRING, HENRY. *See* Manson, James E., 156
EYRING, HENRY, STEWART, GEORGE H., and SMITH, RICHARD P. Principle of Minimum Bending of Localized and Delocalized Orbitals: Ethane Barrier and Related Effects, 259

Factors, incompatibility, in *Schizophyllium commune*, structure of (Raper, Baxter, and Middleton), 889
FAIRHURST, ALAN S. *See* Smith, Robert E., 705
Fick principle, application of, to measurement of pulmonary blood flow (Fritts and Cournand), 1079
FIRSHEIN, WILLIAM, and BRAUN, WERNER. On the Nature of the Selective Effects of Deoxyribonucleic Acid Digest upon Pneumococci of Different Virulence, 918
FISCHER, HERMANN O. L. *See* Baer, Hans Helmut, 991
FISHER, HAROLD W., PUCK, THEODORE T., and SATO, GORDON. Molecular Growth Requirements of Single Mammalian Cells: The Action of Fetuin in Promoting Cell Attachment to Glass, 4
5-Fluorouracil and its derivatives, mode of action of (Cohen, Flaks, Barner, Loeb, and Lichtenstein), 1004
FLAKS, JOEL G. *See* Cohen, Seymour S., 1004
FOLEY, KATHRYN A. *See* Harbury, Henry A., 662

Foundations of the theory of distributions (Gál), 1248
FRAENKEL, GIDEON, and NIEMANN, CARL. Mode of Protonation of Amides, The, 688
FRANCK, JAMES. Remarks on the Long-Wave Limits of Photosynthesis and Chlorophyll Fluorescence, 941
FREDERICKS, R. W. Solution of a Pair of Integral Equations from Elastostatics, 309
FREESE, ERNST. *See* Benzer, Seymour, 112
FRITTS, H. W., JR., and COURNAND, A. Application of the Fick Principle to the Measurement of Pulmonary Blood Flow, The 1079
FRUTON, JOSEPH S. *See* Zioudrou, Christine, 439
FUJII, SETSURO. *See* Zioudrou, Christine, 439
FUJIMORI, E. *See* Tollin, G., 1035
FULLER, R. C., ANDERSON, I. C., and NATHAN, H. A. Pteridines in Photosynthesis, 239
———. Pteridines in Photosynthesis—an Artifact of Paper Chromatography, 518
Functions: algebraic, purity of branch locus of (Zariski), 791; generating, and the semigroup theory of branching Markov processes (Bharucha-Reid and Rubin), 1057
Functors, non-additive, their derived functors, and suspension homomorphism (Dold and Puppe), 1065
FUOSS, RAYMOND M., BERKOWITZ, JOAN B., HIRSCH, ERNEST, and PETRUCCI, SERGIO. Conductance of Tetrabutylammonium Tetraphenylboride, 27

GÁL, I. S. On the Foundations of the Theory of Distributions, 1248
GARING, JOHN S., and NIELSEN, HARALD H. *l*-Type Doubling in NH_3, 467
Garnet locus, nature of, in *D. melanogaster* (Hexter), 768
GAULDEN, MARY ESTHER, and PERRY, ROBERT P. Influence of the Nucleolus on Mitosis as Revealed by Ultraviolet Microbeam Irradiation, 553
GEESLIN, ROGER H. Note on a Class of Diffusion Equations, 788
Gene complexes, coadapted, breakdown of, and interracial hybridization in *D. willistoni* (Dobzhansky and Pavlovsky), 622
GENGOZIAN, N., MAKINODAN, T., CONGDON, C. C., and OWEN, R. D. Immune Status of Long-Term Survivors of Lethally X-irradiated Mice Protected with Isologous, Homologous, or Heterologous Bone Marrow, The, 560
GEORGE, PHILIP, and LYSTER, R. L. J. Crevice Structures in Hemoprotein Reactions, 1013
Germ cells, developing, biological damage in (Alexander), 1217
GEUSIC, J. E. *See* Williams, Dudley, 1127
GILES, NORMAN H. *See* Case, Mary E., 378
———. *See* Woodward, Dow O., 1237
GLASSMAN, EDWARD. *See* Schweet, Richard S., 173
GOLDIE, A. W. Structure of Prime Rings with Maximum Conditions, The, 584
GOLDSMITH, TIMOTHY H. Visual System of the Honeybee, The, 123
GORDY, WALTER. *See* Miyagawa, Ichiro, 613
GOUGH, PHILIP. *See* Hilgard, Ernest R., 1255
GOWER, JOSEPHINE S. *See* Russell, W. L., 901
GRAHAM, C. H., and HSIA, YUN. Spectral Luminosity Curves for a Dichromatic Eye and a Normal Eye in the Same Person, The, 46
GRIFFEN, A. B. Occurrence of Chromosomal Aberrations in Pre-spermatocytic Cells of Irradiated Male Mice, 691
GRIFFIN, BEVERLY E., TODD, SIR ALEXANDER, and RICH, ALEXANDER. Synthesis of Ribothymidine-5′-Pyrophosphate and Its Conversion to Polyribothymidylic Acid, A, 1123
Growth-promoting dislocations, inactivation of, due to temperature, pressure, and poisons (Manson, Cagle, and Eyring), 156
GUILDNER, LESLIE A. Thermal Conductivity of Carbon Dioxide in the Region of the Critical Point, The, 1149

HAAS, F L. *See* Doudney, C. O., 390
HAKKINEN, HERTTA-MAIJA. *See* Williams, Roger J., 216
HALVORSON, HARLYN, DOI, ROY, and CHURCH, BROOKS. Dormancy of Bacterial Endospores: Regulation of Electron Transport by Dipicolinic Acid, 1171
HAND, CADET. *See* Muscatine, Leonard, 1259
HANEDA, YATA, and JOHNSON, FRANK H. Luciferin-Luciferase Reaction in a Fish, *Parapriacanthus beryciformis*, of Newly Discovered Luminescence, The, 127
HARBURY, HENRY A., and FOLEY, KATHRYN A. Molecular Interaction of Isoalloxazine Derivatives, 662
HECHT, L. I. *See* Zamecnik, Paul C., 73
HECHTER, OSCAR. *See* Lester, Gabriel, 1141
Hemoglobin, synthesis of, in cell-free system (Schweet, Lamfrom, and Allen), 1029
Hemoglobins A, S, and C, human adult, dissociation and recombination of (Itano and Singer), 522
Hemoprotein reactions, crevice structures in (George and Lyster), 1013
Heterosis causing increase in fitness in experimental populations (Carson), 1136
Hexose, asymmetric labeling of (Rose), 10
HEXTER, W. M. On the Nature of the Garnet Locus in *Drosophila melanogaster*, 768
HILDEBRANDT, A. C. Stimulation on Inhibition of Virus of Infected and Insect-Gall Tissues and Single-Cell Clones, 353
HILGARD, ERNEST R., WEITZENHOFFER, ANDRÉ M., and GOUGH, PHILIP. Individual Differences in Susceptibility to Hypnosis, 1255
HIRAI, NISHIO. *See* Eyring, Henry, 683, 1213
HIRSCH, ERNEST. *See* Fuoss, Raymond M., 27
HIRSCHBERG, ERICH. *See* Marks, Paul A., 529
HODGE, ALAN J., and SCHMITT, FRANCIS O. Interaction Properties of Sonically Fragmented Collagen Macromolecules, 418
HODGSON, E. S. *See* Ozbas, S., 825

HOLLINGWORTH, BARBARA K. *See* Edsall, John T., 505
Honeybee, visual system of (Goldsmith), 123
HSIA, YUN. *See* Graham, C. H., 46
HUBBARD, RUTH, and KROPF, ALLEN. Action of Light on Rhodopsin, The, 130
HUGHES, W. L., BOND, V. P., BRECHER, G., CRONKITE, E. P., PAINTER, R. B., QUASTLER, H., and SHERMAN, F. G. Cellular Proliferation in the Mouse as Revealed by Autoradiography with Tritiated Thymidine, 476
HURLOCK, BARBARA. *See* Talalay, Paul, 862
HUSAIN, AKHTAR, and DIMOND, A. E. Function of Extracellular Enzymes of Dutch Elm Disease Pathogen, The, 594
Hybridization, interracial, and breakdown of coadapted gene complexes in *D. willistoni* (Dobzhansky and Pavlovsky), 622
Hydrogen transfer between pyridine nucleotides, activation of (Talalay and Williams-Ashman), 15
Hydroxylamine, effects of, on $C^{14}O_2$ fixation pattern during photosynthesis (Bassham, Kirk, and Calvin), 491
Hypnosis, individual differences in susceptibility to (Hilgard, Weitzenhoffer, and Gough), 1255

IGUSA, JUN-ICHI. Class Number of a Definite Quaternion with Prime Discriminant, 312
———. *See* Chow, Wei-Liang, 1244
ILLINGWORTH, BARBARA, JANSZ, HENDRIK S., BROWN, DAVID H., and CORI, CARL F. Observations on the Function of Pyridoxal-5-Phosphate in Phosphorylase, 1180
IMAHORI, K. *See* Doty, P., 424
Imbedding, invariant, wave propagation, and the WKB approximation (Bellman and Kalaba), 317
Induction, irradiation-elicited, transfer of (Borek and Ryan), 374
Integral, surface, concept of (Cesari and Turner), 42
Intraperitoneal injections of DNA preparations followed by cytological and cytochemical changes in livers of white mice (Leuchtenberger, Leuchtenberger, and Uyeki), 700
Invariance of openness (Whyburn), 464
Invariance, principles of, and invariant imbedding relation (Preisendorfer), 320; time-dependent principles of (Preisendorfer), 328
Irradiation damage in crystalline carbohydrates, paramagnetic resonance study of (Williams, Geusic, Wolfrom, and McCabe), 1127
ISENBERG, IRVIN, and SZENT-GYÖRGYI, ALBERT. Free Radical Formation in Riboflavin Complexes, 857
———. On the Emission of Tryptophan, 519
Isoalloxazine derivatives, molecular interaction of (Harbury and Foley), 662
Isoleucine and valine, biosynthesis of, in *N. crassa* (Wagner, Radhakrishnan, and Snell), 1047
ITANO, HARVEY A., and SINGER, S. J. On Dissociation and Recombination of Human Adult Hemoglobins A, S, and C, 522
IYER, V. J., and SZYBALSKI, WACLAW. Mechanism of Chemical Mutagenesis, The. I. Kinetic Studies on the Action of Triethylene Melamine (TEM) and Azaserine, 446

JANSZ, HENDRIK S. *See* Illingworth, Barbara, 1180
JOHNSON, ANNE B. *See* Marks, Paul A., 529
JOHNSON, FRANK H. *See* Haneda, Yata, 127

KALABA, ROBERT. *See* Bellman, Richard, 317
KAPLAN, JOSEPH, and BARTH, CHARLES A. Chemical Aeronomy, 105
KAPLAN, NATHAN O. *See* Shifrin, Sidney, 177
KARREMAN, GEORGE, STEELE, RICHARD H., and SZENT-GYÖRGYI, ALBERT. On Resonance Transfer of Excitation Energy between Aromatic Amino Acids in Proteins, 140
KATZ, EDWARD. *See* Waksman, Selman A., 602
KAUS, PETER. *See* Pittendregh, Colin, 965
KERVAIRE, MICHEL A. Non-Parallelizability of the n-Sphere for $n > 7$, 280
KINGDON, HENRY S., WEBSTER, LESLIE T., JR., and DAVIE, EARL W. Enzymatic Formation of Adenyl Tryptophan: Isolation and Identification, 757
KIRK, M. *See* Bassham, J. A., 491
KLEIN, RICHARD M. Activation of Metabolic Systems during Tumor-Cell Formation, 350
KLEMPERER, E. *See* Doty, P., 424
KLINGENBERG, W. On the Structure of Compact Riemannian Manifolds, 586
KOPPELMAN, WALTER. On the Spectral Theory of Singular Integral Operators, 1252
KORNBERG, ARTHUR. *See* Adler, Julius, 641
———. *See* Bessman, Maurice J., 633
———. *See* Lehman, I. R., 1191
KOSHLAND, D. E., JR. Application of a Theory of Enzyme Specificity to Protein Synthesis, 98
KOSTANT, BERTRAM. Formula for the Multiplicity of a Weight, A, 588
KROPF, ALLEN. *See* Hubbard, Ruth, 130

LAHA, R. G. Example of a Nonnormal Distribution where the Quotient Follows the Cauchy Law, An, 222
LAMFROM, HILDEGARDE. *See* Schweet, Richard, 1029
LARKS, S. D. Human Electrohysterogram: Wave Forms and Implications, The, 820
LAW, J. *See* Chaykin, S., 998
LEHMAN, I. R. *See* Adler, Julius, 641
———. *See* Bessman, Maurice J., 633
LEHMAN, I. R., ZIMMERMAN, STEVEN B., ADLER, JULIUS, BESSMAN, MAURICE J., SIMMS, E. S., and KORNBERG, ARTHUR. Enzymatic Synthesis of Deoxyribonucleic Acid. V. Chemical Composition of Enzymatically Synthesized Deoxyribonucleic Acid, 1191
LEHMAN, SHERMAN. *See* Bellman, Richard, 905
LESTER, GABRIEL, and HECHTER, OSCAR. Dissociation of Rubidium Uptake by

Neurospora crassa into Entry and Binding Phases, 1141
LEUCHTENBERGER, CECILIE, LEUCHTENBERGER, RUDOLF, and UYEKI, EDWIN. Cytological and Cytochemical Changes in Livers of White Mice Following Intraperitoneal Injections of DNA Preparations from Breast Cancers of Agouti C3H Mice, 700
LEUCHTENBERGER, RUDOLF. *See* Leuchtenberger, Cecilie, 700
LIBBY, W. F. Radioactive Fallout, 800
LICHTENSTEIN, JANET. *See* Cohen, Seymour S., 1004
Light, action on rhodopsin (Hubbard and Kropf), 130
Light emission, delayed, in green plant materials (Tollin, Fujimori, and Calvin), 1035
Linkage of single deoxynucleotides to the deoxynucleoside ends of DNA (Adler Lehman, Bessman, Simms, and Kornberg), 641
LIPMANN, FRITZ. Symposium on Amino Acid Activation. Chairman's Introduction: Some Facts and Problems, 67
———. *See* Mager, J., 305
———. *See* Weiss, Samuel B., 189
———. *See* Zachau, Hans Georg, 885
LIPPINCOTT, BARBARA B. *See* Commoner, Barry, 1099, 1110
Liquid state, significant structures in (Eyring, Ree, and Hirai), 683
LITT, MICHAEL, MARMUR, JULIUS, EPHRUSSI-TAYLOR, HARRIETT, and DOTY, PAUL. Dependence of Pneumococcal Transformation on the Molecular Weight of Deoxyribose Nucleic Acid, The, 144
LOCKINGEN, LLOYD S. Effects of Reduced Oxygen Tension on Growth Responses of Methionine and Vitamin B_{12} Stimulated Mutants of Coliform Bacteria, 924
LOEB, MARILYN R. *See* Cohen, Seymour S., 1004
Longevity and obesity, relation between, in human beings (Pauling), 619
Luciferin-luciferase reaction in a fish (Haneda and Johnson), 127
LURIA, S. E. *See* Adams, Jane N., 590
LUXORO, MARIO. Observations in Myelin Structure: Incisures and Nodal Regions, 152
LYSTER, R. L. J. *See* George, Philip, 1013

MCCABE, LEO J. *See* Williams, Dudley, 1127
MCCONNELL, HARDEN M. Pseudovector Nuclear Hyperfine Interaction, A, 766
MCGILL, BARBARA BUNCE. *See* Doty, Paul, 432
MCLACHLAN, DAN, JR. Crystal Structure and Information Theory, 948
MADDUX, SAVANNA C. *See* Russell, W. L., 901
MAGER, J., and LIPMANN, FRITZ. Amino Acid Incorporation and the Reversion of Its Initial Phase with Cell-Free *Tetrahymena* Preparations, 305
Magnetic fields, force-free (Chandrasekhar and Woltjer), 285; force free, theorem on (Woltjer), 489
MAKINODAN, T. *See* Gengozian, N., 560
Mammalian cells, molecular growth requirements of (Fisher, Puck, and Sato), 4
MANSON, JAMES E., CAGLE, F. W., JR., and EYRING, HENRY. Inactivation of Growth-promoting Dislocations with Temperature, Pressure, and Poisons, The, 156
Mappings, differentiable, in Schoenflies problem (Morse), 1068
MARKS, PAUL A., JOHNSON, ANNE B., and HIRSCHBERG, ERICH Effect of Age on the Enzyme Activity in Erythrocytes, 529
MARMUR, JULIUS. *See* Litt, Michael, 144
MARTIN, R. BRUCE. *See* Edsall, John T., 505
Materials, transfer of, from symbiotic algae to tissues of a coelenterate (Muscatine and Hand), 1259
Measures orthogonal to polynomials (Bishop), 278
Membranes and plates, additional note (Szegö), 314
MESCHIA, G., WOLKOFF, A. S., and BARRON, D. H. Difference in Electric Potential across the Placenta of Goats, 483
MESELSON, MATTHEW, and STAHL, FRANKLIN W. Replication of DNA in *Escherichia coli*, The, 671
METZNER, BARBARA. *See* Metzner, Helmut, 205
METZNER, HELMUT, METZNER, BARBARA, and CALVIN, MELVIN. Early Unstable CO_2 Fixation Products in Photosynthesis, 205
Mice, lethally X-irradiated, immune status of long-term survivors (Gengozian, Makinodan, Congdon, and Owen), 560
Micro-organisms, protein turnover in (Borek, Ponticorvo, and Rittenberg), 369
MIDDLETON, RICHARD B. *See* Raper, John R., 899
MIRSKY, A. E. *See* Allfrey, V. G., 981
Mitosis, influence of nucleolus on (Gaulden and Perry), 553
MIYAGAWA, ICHIRO, GORDY, WALTER, WATABE, NORIMITSU, and WILBUR, KARL M. On the Nature of Free Radicals Detected by Paramagnetic Resonance in Biological Substances, 613
Molecular interaction of isoalloxazine derivatives (Harbury and Foley), 662
Molecules, potential barriers to internal rotation (Pauling), 211
MORSE, MARSTON. Differentiable Mappings in the Schoenflies Problem, 1068
MOSES, V., and CALVIN, M. Path of Carbon in Photosynthesis, The. XXII. The Identification of Carboxy-ketopentitol Diphosphates as Products of Photosynthesis, 260
MUELLER, HELMUT. Dehydrated Muscle Fibers, 235
———. *See* Arnold, William, 1
MUSCATINE, LEONARD, and HAND, CADET. Direct Evidence for the Transfer of Materials from Symbiotic Algae to the Tissues of a Coelenterate, 1259
Muscle fibers: dehydrated (Mueller), 235; magnetic asymmetry of (Arnold, Steele, and Mueller), 1
Mutagenesis, chemical, mechanism of (Iyer and Szybalski), 446
Mutation rates in female mice, radiation-in-

duced (Russell, Russell, Gower, and Maddux), 901
Mutations, induced by 5-bromouracil (Benzer and Freese), 112
Myelin structure: incisures and nodal regions (Luxoro), 152

n-Sphere, non-parallelizability of, for $n > 7$ (Kervaire), 280
NACHBIN, LEOPOLDO. On the Operational Calculus with Differentiable Functions, 698
NAGATA, MASAYOSHI. Remarks on a Paper of Zariski on the Purity of Branch Loci, 796
NANNEY, D. L. Epigenetic Control Systems, 712
NATHAN, H. A. *See* Fuller, R. C., 239, 518
NATIONAL ACADEMY OF SCIENCES: consciousness, mechanisms of (Penfield), 51; officers, council, members, foreign associates, sections, geographical listing, 719; symposium on amino acid activation (Lipmann), 67; symposium on plant tumors (Riker), 338
Neurosecretion, action of, upon central nervous system in vitro and upon behavior (Ozbas and Hodgson), 825
NICOL, C. A., and VANDIVER, H. S. Supplement to a Paper Entitled "A Von Sterneck Arithmetical Function and Restricted Partitions with Respect to a Modulus," 917
NIELSEN, HARALD H. *See* Garing, John S., 467
NIEMANN, CARL. *See* Fraenkel, Gideon, 688
Night blindness and vitamin A deficiency (Dowling and Wald), 648
NISHIHARA, TOMIO, and DOTY, PAUL. Sonic Fragmentation of Collagen Macromolecules, The, 411
NIU, M. C. Thymus Ribonucleic Acid and Embryonic Differentiation, 1264
Nonnormal distribution, example where quotient follows Cauchy Law (Laha), 222
NORTHROP, JOHN H. Concerning the Origin of Bacterial Viruses, 229
NOVELLI, G. DAVID. Some Problems Concerning the Activation of Amino Acids, 86
Nucleation, spontaneous, in supersaturated vapors (Rodebush and Clarke), 536
Nucleolus, influence of, on mitosis (Gaulden and Perry), 553
Nucleotides, pyridine, hydrogen transfer between (Talalay and Williams-Ashman), 15

Obesity, relation between longevity and, in human beings (Pauling), 619
OCHOA, SEVERO. *See* Beljanski, Mirko, 494, 1157
OFENGAND, E. J. *See* Berg, P., 78
OLIVER, JACK, and EWING, MAURICE. Seismic Surface Waves at Palisades from Explosions in Nevada and the Marshall Islands, 780
Openness, invariance of (Whyburn), 464
Operators: normal, spectral representation of semigroups of (Tulcea), 44; singular integral, spectral theory of (Koppelman), 1252; R and T, functional relations for, on plane-parallel media (Preisendorfer) 323
Orbitals, localized and delocalized, minimum bending of (Eyring, Stewart, and Smith), 259
OWEN, R. D. *See* Gengozian, N., 560
Oxygen tension, reduced, effects on growth responses of methionine and vitamin B_{12} stimulated mutants of coliform bacteria (Lockingen), 924
OZBAS, S., and HODGSON, E. S. Action of Neurosecretion upon Central Nervous System in Vitro and upon Behavior, 825

PAINTER, R. B. *See* Hughes, W. L., 476
PARTRIDGE, C. W. H. *See* Woodward, Dow O., 1237
PASSONNEAU, JANET V. *See* Commoner, Barry, 1099
PAULING, LINUS. Nature of Bond Orbitals and the Origin of Potential Barriers to Internal Rotation in Molecules, The, 211
———. Relation between Longevity and Obesity in Human Beings, The, 619
PAVLOVSKY, OLGA. *See* Dobzhansky, Theodosius, 622
PELTON, RICHARD B. *See* Williams, Roger J., 216
PENFIELD, WILDER. Some Mechanisms of Consciousness Discovered during Electrical Stimulation of the Brain, 51
PERRY, ROBERT P. *See* Gaulden, Mary Esther, 553
PETRUCCI, SERGIO. *See* Fuoss, Raymond M., 27
Phase transitions, optimum conditions for (Eyring, Cagle, and Christensen), 120
PHILLIPS, A. H. *See* Chaykin, S., 998
Phosphorylase, function of pyridoxal-5-phosphate in (Illingworth, Jansz, Brown, and Cori), 1180
Phosphorylated intermediates in synthesis of squalene (Chaykin, Law, Phillips, Tchen, and Bloch), 998
Photosynthesis and chlorophyll fluorescence, long-wave limits of (Franck), 941
Photosynthesis: effects of hydroxylamine on $C^{14}O_2$ fixation pattern during (Bassham, Kirk, and Calvin), 491; path of carbon in (Moses and Calvin), 260; pteridines in (Fuller, Anderson, and Nathan), 239, 518; unstable CO_2 fixation products in (Metzner, Metzner, and Calvin), 205
PITTENDREGH, COLIN ,BRUCE, VICTOR, and KAUS, PETER. On the Significance of Transients in Daily Rhythms, 965
Pneumococcal transformation, dependent on molecular weight of DNA (Litt, Marmur, Ephrussi-Taylor, and Doty), 144
Pneumococci of different virulence, selective effects of DNA digest upon (Firshein and Braun), 918
Polymers, high, viscosity of (Eyring, Ree, and Hirai), 1213
Polynomials, measures orthogonal to (Bishop), 278
Polyribothymidylic acid, converted from a synthesis of ribothymidine-5′-pyrophosphate (Griffin, Todd, and Rich), 1123
PONTICORVO, LAURA. *See* Borek, Ernest, 369

Populations, experimental, increase in fitness in, resulting from heterosis (Carson), 1136
Post-irradiation treatment to modify ultraviolet-induced mutation frequency and survival in bacteria (Doudney and Haas), 390
PREISENDORFER, RUDOLPH W. Functional Relations for the R and T operators on Plane-parallel Media, 323
———. Invariant Imbedding Relation for the Principles of Invariance, 320
———. Time-dependent Principles of Invariance, 328
Programming, dynamic, successive approximation, and monotone convergence (Bellman), 578
Protein biosynthesis by a cell-free bacterial system (Beljanski and Ochoa), 494, 1157
Protein components of tryptophan synthetase of *E. coli* (Crawford and Yanofsky), 1161
Protein turnover in micro-organisms (Borek, Ponticorvo, and Rittenberg), 369
Proteins: excited states of (Steele and Szent-Györgyi), 540; labeling of, by isotopic amino acid derivatives (Zioudrou, Fujii, and Fruton), 439; resonance transfer of excitation energy in (Karreman, Steele, and Szent-Györgyi), 140; thiolation of (Benesch and Benesch), 848
Protoplast-like structures, production reproduction and reversion of, in osmotic strain of *N. crassa* (Emerson and Emerson), 668
Pseudoallelism at the notch locus of *D. melanogaster* (Welshons), 254
Pseudovector nuclear hyperfine interaction (McConnell), 766
Pteridines in photosynthesis (Fuller, Anderson, and Nathan), 239, 518
PUCK, T. T. *See* Tjio, J. H., 1229
PUCK, THEODORE T. Action of Radiation on Mammalian Cells. III. Relationship between Reproductive Death and Induction of Chromosome Anomalies by X-Irradiation of Euploid Human Cells in vitro, 772
———. *See* Fisher, Harold W., 4
PULLMAN, ALBERTE. *See* Pullman, Bernard, 1197
PULLMAN, BERNARD, and PULLMAN, ALBERTE. Electron-Donor and -Acceptor Properties of Biologically Important Purines, Pyrimidines, Pteridines, Flavins, and Aromatic Amino Acids, 1197
Pulmonary blood flow, application of Fick principle to measurement of (Fritts and Cournand), 1079
PUPPE, DIETER. *See* Dold, Albrecht, 1065
Pyridoxal-5-phosphate in phosphorylase, function of (Illingworth, Jansz, Brown, and Cori), 1180
Pyrimidine and purine analogues, incorporation of, into DNA (Bessman, Lehman, Adler, Zimmerman, Simms, and Kornberg), 633

QUASTLER, H. *See* Hughes, W. L., 476
Quaternion, definite, with prime discriminant, class number of (Igusa), 312

RADHAKRISHNAN, A. N. *See* Wagner, R. P., 1047
Radiation: action of, on mammalian cells (Puck), 772; diffuse reflection of a pencil of, by a plane-parallel atmosphere (Chandrasekhar), 933
Radiation-induced mutation rates in female mice (Russell, Russell, Gower, and Maddux), 901
Radical formation, free, in riboflavin complexes (Isenberg and Szent-Györgyi), 857
Radicals, free: detected by paramagnetic resonance in biological substances, nature of (Miyagawa, Gordy, Watabe, and Wilbur), 613; light-induced, in FMN and flavoprotein enzymes (Commoner and Lippincott), 1110
Radioactive fallout (Libby), 800
RAHM, WALTER E. *See* Wever, Ernest Glen, 1087
RAFER, JOHN R., BAXTER, MARGERY G., and MIDDLETON, RICHARD B. Structure of the Incompatibility Factors in *Schizophyllium commune*, The, 889
Recombination, normal and aberrant, between allelic mutants in *N. crassa* (Case and Giles), 378
REE, TAIKYUE. *See* Eyring, Henry, 683, 1213
Reflection, diffuse, of a pencil of radiation by a plane-parallel atmosphere (Chandrasekhar), 933
Relations, functional, for the R and T operators on plane-parallel media (Preisendorfer), 323
Relationship between reproductive death and induction of chromosome anomalies by X-irradiation of euploid human cells in vitro (Puck), 772
Relativistic field theory, Euclidean structure of (Schwinger), 956
Resonance studies, electron-spin, of free-radical intermediates in oxidation-reduction enzyme systems (Commoner, Lippincott, and Passonneau), 1099
Resonance study, paramagnetic, of irradiation damage in crystalline carbohydrates (Williams, Geusic, Wolfrom, and McCabe), 1127
Rhythms, daily, significance of transients in (Pittendrigh, Bruce, and Kaus), 965
Riboflavin complexes, free radical formation in (Isenberg and Szent-Györgyi), 857
RNA: extracted from thymus and embryonic differentiation (Niu), 1264; incorporation of amino acids into (Schweet, Bovard, Allen, and Glassman), 173
RICE, STUART A. *See* Doty, Paul, 432
RICH, ALEXANDER. *See* Griffin, Beverly E., 1123
RICHARDS, FREDERIC M. On the Enzymic Activity of Subtilisin-modified Ribonuclease, 162
Riemannian manifolds, compact, structure of (Klingenberg), 586
RIKER, A. J. Conclusions (Symposium on Plant Tumors), 367
———. Introduction to the Symposium on Plant Tumors, 338
RILLING, H., TCHEN, T. T., and BLOCH, KONRAD. On the Mechanism of Squalene Biogenesis, 167

Rings, prime, with maximum conditions, structure of (Goldie), 584
RITTENBERG, D. *See* Borek, Ernest, 369
ROBBINS, WILLIAM J. Sucrose and Growth of Excised Roots of an Inbred *Zea mays*, 1210
RODEBUSH, WORTH H., and CLARKE, JOHN V., JR. Spontaneous Nucleation in Supersaturated Vapors, 536
ROGERS, LORENE L. *See* Williams, Roger J., 216
ROSE, IRWIN A. Mechanism of Action of Aldolase and the Asymmetric Labeling of Hexose, The, 10
ROSENBERG, BARBARA H. *See* Cavalieri, Liebe F., 853
Rubidium uptake, dissociation of, by *N. crassa* into entry and binding phases (Lester and Hechter), 1141
RUBIN, HERMAN. *See* Bharucha-Reid, A. T., 1057
RUSSELL, LIANE BRAUCH. *See* Russell, W. L., 901
RUSSELL, W. L., RUSSELL, LIANE BRAUCH, GOWER, JOSEPHINE S., and MADDUX, SAVANNA C. Radiation-induced Mutation Rates in Female Mice, 901
RYAN, ANN. *See* Borek, Ernest, 374

SATO, GORDON. *See* Fisher, Harold W., 4
SCHMITT, FRANCIS O. *See* Hodge, Alan J., 418
SCHWEET, RICHARD S., BOVARD, FREEMAN C., ALLEN, ESTHER, and GLASSMAN, EDWARD. Incorporation of Amino Acids into Ribonucleic Acid, The, 173
SCHWEET, RICHARD, LAMFROM, HILDEGARDE, and ALLEN, ESTHER. Synthesis of Hemoglobin in a Cell-free System, The, 1029
SCHWINGER, JULIAN. Addendum to Spin, Statistics, and the TCP Theorem, 617
———. On the Euclidean Structure of Relativistic Field Theory, 956
———. Spin, Statistics, and the TCP Theorem, 223
Segregation and pseudoallelism, aberrant, at the garnet locus in *D. melanogaster* (Chovnick), 333
Seismic surface waves at Palisades from explosions in Nevada and Marshall Islands (Oliver and Ewing), 780
Semiradical of a semiring (Bourne and Zassenhaus), 907
Semiring, semiradical of (Bourne and Zassenhaus), 907
SENO, TAMIKAZU, and BÜYÜKÖZER, ILHAN. Cartilage Formation in Somite Grafts of Chick Blastoderm, 1274
Serotonin, probable mechanism of action of (Woolley), 197
Serotonin receptors (Woolley), 1202
SESHADRI, C. S. Triviality of Vector Bundles over the Affine Space K^2, 456
SHEARER, GEORGIA B. *See* Commoner, Barry, 1117
SHERMAN, F. G. *See* Hughes, W. L., 476
SHIFRIN, SIDNEY, and KAPLAN, NATHAN O. Fluorescence Studies of Coenzyme Binding to Dehydrogenases, 177
SIMMS, E. S. *See* Adler, Julius, 641
———. *See* Bessman, Maurice J., 633
———. *See* Lehman, I. R., 1191
SINGER, S. J. *See* Itano, Harvey A., 522
SMITH, RICHARD P. *See* Eyring, Henry, 259
SMITH, ROBERT E., and FAIRHURST, ALAN S. Mechanism of Cellular Thermogenesis in Cold-Adaptation, A, 705
SMYTH, R. D. *See* Barker, H. A., 1093
SNELL, E. E. *See* Wagner, R. P., 1047
Sounds, high-frequency, cochlear potentials in the cat in response to (Wever, Vernon, Rahm, and Strother), 1087
Space-curve boundary, surface of (Evans), 786
Spaces for which Ricci scalar R is equal to zero (Eisenhart), 695
Spectral luminosity curves for a dichromatic eye and for a normal eye (Graham and Hsia), 46
Spectral theory of singular integral operators (Koppelman), 1252
Spin, statistics, and TCP theorem (Schwinger), 223; addendum to (Schwinger), 617
SPIZIZEN, JOHN. Transformation of Biochemically Deficient Strains of *Bacillus subtilis* by Deoxyribonucleate, 1072
SPRINGER, GEORG F., and ANSELL, NORMA J. Inactivation of Human Erythrocyte Agglutinogens M and N by Influenza Viruses and Receptor-destroying Enzyme, 182
Squalene biogenesis, mechanism of (Rilling, Tchen, and Bloch), 167
STADLER, JOAN. *See* Yanofsky, Charles, 245
STAHL, FRANKLIN W. *See* Meselson, Matthew, 671
Statistics, spin and TCP theorem (Schwinger), 223
STEELE, RICHARD. *See* Arnold, William, 1
STEELE, RICHARD H. *See* Karreman, George, 140
STEELE, RICHARD H., and SZENT-GYÖRGYI, ALBERT. Studies on the Excited States of Proteins, 540
STEPHENSON, M. L. *See* Zamecnik, Paul C., 73
STEWART, GEORGE H. *See* Eyring, Henry, 259
STONE, WILSON S., and WILSON, FLORENCE D. Genetic Studies of Irradiated Natural Populations of *Drosophila*. II. 1957 Tests, 565
STRODE, CLARICE. *See* Commoner, Barry, 1117
Strong Markov theorem of Chung, new proof of (Austin), 575
STROTHER, WILLIAM F. *See* Wever, Ernest Glen, 1087
Structures, crevice, in hemoprotein reactions (George and Lyster), 1013
STULBERG, M. P. *See* Boyer, P. D., 92
Subtilisin-modified ribonuclease, enzymic activity of (Richards), 162
Sucrose and growth of excised roots of an inbred *Zea mays* (Robbins), 1210
Sugars, 3-amino, new way for synthesis of (Baer and Fischer), 991
Surface integral, concept of (Cesari and Turner), 42
SUSSMAN, MAURICE. *See* Ennis, Herbert L., 401

Symbiotic algae, transfer of materials from, to tissues of a coelenterate (Muscatine and Hand), 1259
Synthesis: enzymatic, of fatty acids by aldol condensation (Brady), 993; of hemoglobin in cell-free system (Schweet, Lamfrom, and Allen), 1029; of ribothymidine-5′-pyrophosphate and its conversion to polyribothymidylic acid (Griffin, Todd, and Rich), 1123; of squalene, phosphorylated intermediates in (Chaykin, Law, Phillips, Tchen, and Bloch), 998; of 3-amino sugars, a new way for (Baer and Fischer), 991
SZEGÖ, G. Note to My Paper "On Membranes and Plates," 314
SZENT-GYÖRGYI, ALBERT. *See* Isenberg, Irvin, 519, 857
———. *See* Karreman, George, 140
———. *See* Steele, Richard H., 540
SZYBALSKI, WACLAW. *See* Iyer, V. J., 446

TCP theorem, spin and statistics (Schwinger), 223
TALALAY, PAUL, HURLOCK, BARBARA, and WILLIAMS-ASHMAN, H. G. On a Coenzymatic Function of Estradiol-17-β, 862
TALALAY, PAUL, and WILLIAMS-ASHMAN, H. G. Activation of Hydrogen Transfer between Pyridine Nucleotides by Steroid Hormones, 15
TCHEN, T. T. *See* Chaykin, S., 998
———. *See* Rilling, H., 167
Tetrabutylammonium tetraphenylboride, conductance of (Fuoss, Berkowitz, Hirsch, and Petrucci), 27
Tetrapod extinctions at the end of the Triassic period (Colbert), 973
Thermogenesis, cellluar, mechanism of, in cold-adaptation (Smith and Fairhurst), 705
TJIO, J. H., and PUCK, T. T. Somatic Chromosomes of Man, The, 1229
Tobacco mosaic virus, linear analysis of (Commoner, Shearer, and Strode), 1117
TODD, SIR ALEXANDER. *See* Griffin, Beverly E., 1123
TOLLIN, G., FUJIMORI, E., and CALVIN, M. Delayed Light Emission in Green Plant Materials: Temperature-Dependence and Quantum Yield, 1035
TOWNSEND, J. IVES. Chromosomal Polymorphism in Caribbean Island Populations of *Drosophila willistoni*, 38
Traffic flow, principles for simulating (Charnes and Cooper), 201
Transients in daily rhythms, significance of (Pittendregh, Bruce, and Kaus), 965
Tryptophan, emission of (Isenberg and Szent-Györgyi), 519
Tryptophan synthetase: enzymatic activity of the immunologically related protein (Yanofsky and Stadler), 245; of *E. coli* separated into two protein components (Crawford and Yanofsky), 1161
TULCEA, CASSIUS IONESCU. Spectral Representation of Semigroups of Normal Operators, 44
Tumors, plant, symposium on (Riker), 338
TURNER, L. H. *See* Cesari, L., 42

UYEKI, EDWIN. *See* Leuchtenberger, Cecilie, 700

VANDIVER, H. S. Rapid Computing Machine as an Instrument in the Discovery of New Relations in the Theory of Numbers, The, 459
———. *See* Nicol, C. A., 917
Vapors, super-saturated, spontaneous nucleation in (Rodebush and Clarke), 536
Varieties over rings, cohomology theory of (Chow and Igusa), 1244
Vector bundles, triviality of, over affine space K^2 (Seshadri), 456
Vector spaces over ordered fields, strong Minkowski-Farkas-Weyl theorem for (Charnes and Cooper), 914
VERNON, JACK A. *See* Wever, Ernest Glen, 1087
VINING, LEO C. *See* Waksman, Selman A., 602
Viruses: bacterial, origin of (Northrop), 229; influenza, effect on human erythrocyte agglutinogens (Springer and Ansell), 182
Vitamin A deficiency and night blindness (Dowling and Wald), 648
Vitamin K, essential for photochemical activity of chloroplasts (Bishop), 501

WAGNER, R. P., RADHAKRISHNAN, A. N., and SNELL, E. E. Biosynthesis of Isoleucine and Valine in *Neurospora crassa*, The, 1047
WAKSMAN, SELMAN A., KATZ, EDWARD, and VINING, LEO C. Nomenclature of the Actinomycins, 602
WALD, GEORGE. *See* Dowling, John E., 648
WATABE, NORIMITSU. *See* Miyagawa, Ichiro, 613
WEBB, H. M. *See* Brown, Frank A., Jr., 290
WEBSTER, LESLIE T., Jr. See Kingdon, Henry S., 757
Weight, formula for multiplicity of (Kostant), 588
WEISS, SAMUEL B., ACS, GEORGE, and LIPMANN, FRITZ. Amino Acid Incorporation in Pigeon Pancreas Fractions, 189
WEISSBACH, H. *See* Barker, H. A., 1093
WEITZENHOFFER, ANDRÉ M. *See* Hilgard, Ernest R., 1255
WELSHONS, W. J. Preliminary Investigation of Pseudoallelism at the Notch Locus of *Drosophila melanogaster*, A, 254
WEVER, ERNEST GLEN, VERNON, JACK A., RAHM, WALTER E., and STROTHER, WILLIAM F. Cochlear Potentials in the Cat in Response to High-Frequency Sound, 1087
WHITE, PHILIP R. Tree Tumor of Unknown Origin, A, 339
WHYBURN, GORDON T. On the Invariance of Openness, 464
WILBUR, KARL M. *See* Miyagawa, Ichiro, 613
WILLIAMS, DUDLEY, GEUSIC, J. E., WOLFROM, M. L., and MCCABE, LEO J. Paramagnetic Resonance Study of Irradiation Damage in Crystalline Carbohydrates. 1127

WILLIAMS, ROGER J., PELTON, RICHARD B., HAKKINEN, HERTTA-MAIJA, and ROGERS, LORENE L. Identification of Blood Characteristics Common to Alcoholic Males, 216
WILLIAMS-ASHMAN, H. G. *See* Talalay, Paul, 15, 862
WILSON, FLORENCE D. *See* Stone, Wilson S., 565
WILSON, P. W. See Esposito, Raymond G., 472
WOLFROM, M. L. *See* Williams, Dudley, 1127
WOLKOFF, A. S. *See* Meschia, G., 483
WOLTJER, L. On Hydromagnetic Equilibrium, 833
———. *See* Chandrasekhar, S., 285
———. Theorem on Force-free Magnetic Fields, A, 489
WOODWARD, DOW O., PARTRIDGE, C. W. H., and GILES, NORMAN H. Complementation at the AD-4 Locus in *Neurospora crassa*, 1237
WOOLLEY, D. W. Probable Mechanism of Action of Serotonin, A, 197
———. Serotonin Receptors. I. Extraction and Assay of a Substance Which Renders Serotonin Fat-Soluble, 1202
Word problem (Boone), 1061

YANOFSKY, CHARLES. *See* Crawford, Irving P., 1161
YANOFSKY, CHARLES, and STADLER, JOAN. Enzymatic Activity Associated with the Protein Immunologically Related to Tryptophan Synthetase, The, 245

ZACHAU, HANS GEORG, ACS, GEORGE, and LIPMANN, FRITZ. Isolation of Adenosine Amino Acid Esters from a Ribonuclease Digest of Soluble, Liver Ribonucleic Acid, 885
ZAMECNIK, PAUL C., STEPHENSON, M. L., and HECHT, L. I. Intermediate Reactions in Amino Acid Incorporation, 73
ZARISKI, OSCAR. On the Purity of the Branch Locus of Algebraic Functions, 791
ZASSENHAUS, HANS. *See* Bourne, Samuel, 907
ZIMMERMAN, STEVEN B. *See* Bessman, Maurice J., 633
———. *See* Lehman, I. R., 1191
ZIOUDROU, CHRISTINE, FUJII, SETSURO, and FRUTON, JOSEPH S. Labeling of Proteins by Isotopic Amino Acid Derivatives, 439

Proceedings of the

National Academy of Sciences

of the United States of America

VOLUME 44, 1958

EDITORIAL BOARD OF THE PROCEEDINGS

WENDELL M. STANLEY
Chairman

HUGH L. DRYDEN, *Home Secretary*
WILLIAM J. ROBBINS, *Treasurer*
H. P. ROBERTSON, *Foreign Secretary*
EDWIN B. WILSON, *Managing Editor*

GREGORY BREIT
HARRISON BROWN
ERNST CLOOS
THOMAS FRANCIS, JR.
C. H. GRAHAM
SAUNDERS MAC LANE
H. W. MAGOUN
HARLOW SHAPLEY
PAUL WEISS

★

PUBLISHED MONTHLY

CONTENTS

NATIONAL ACADEMY OF SCIENCES

Page

AUTUMN MEETING, 1957—

Lecture: Some Mechanisms of Consciousness Discovered During Electrical Stimulation of the Brain . . . . . *By Wilder Penfield* 51
Symposium on Amino Acid Activation . . . . . *Fritz Lipmann, Chairman* 67
Symposium on Plant Tumors . . . . . *A. J. Riker, Chairman* 338

ORGANIZATION
Officers, Council, Members, Members Emeriti, Foreign Associates, Sections, Geographical Listing 719

MATHEMATICS

On the Concept of Surface Integral . . . . . *By L. Cesari and L. H. Turner* 42
Spectral Representation of Semigroups of Normal Operators . . . . . *By Cassius Ionescu Tulcea* 44
Extremal Principles for Simulating Traffic Flow in a Network . . . . . *By A. Charnes and W. W. Cooper* 201
An Example of a Nonnormal Distribution Where the Quotient Follows the Cauchy Law . . . . . *By R. G. Laha* 222
Measures Orthogonal to Polynomials . . . . . *By Errett Bishop* 278
Non-Parallelizability of the n-Sphere for $n > 7$ . . . . . *By Michel A. Kervaire* 280
Solution of a Pair of Integral Equations from Elastostatics . *By R. W. Fredricks* 309
Class Number of a Definite Quaternion with Prime Discriminant . . . . . *By Jun-Ichi Igusa* 312
Note to My Paper "On Membranes and Plates" . . . . . *By G. Szegö* 314
Triviality of Vector Bundles over the Affine Space K^2 . . . . *By C. S. Seshadri* 456
The Rapid Computing Machine as an Instrument in the Discovery of New Relations in the Theory of Numbers . . . . . *By H. S. Vandiver* 459
On the Invariance of Openness . . . . . *By Gordon T. Whyburn* 464
A New Proof of the Strong Markov Theorem of Chung . . . . . *By D. G. Austin* 575
Dynamic Programming, Successive Approximation, and Monotone Convergence . . . . . *By Richard Bellman* 578
On the Theorem of Bertini for Local Domains . . . . . *By Wei-Liang Chow* 580
The Structure of Prime Rings with Maximum Conditions . . . . *By A. W. Goldie* 584
On the Structure of Compact Riemannian Manifolds . . . . . *By W. Klingenberg* 586
A Formula for the Multiplicity of a Weight . . . . . *By Bertram Kostant* 588
Spaces for Which the Ricci Scalar R is Equal to Zero . . . *By Luther P. Eisenhart* 695
On the Operational Calculus with Differentiable Functions *By Leopoldo Nachbin* 698
Surface of Given Space-Curve Boundary . . . . . *By G. C. Evans* 786
Note on a Class of Diffusion Equations . . . . . *By Roger H. Geeslin* 788
On the Purity of the Branch Locus of Algebraic Functions . . *By Oscar Zariski* 791
Remarks on a Paper of Zariski on the Purity of Branch Loci . . . . . *By Masayoshi Nagata* 796
Functional Equations in the Theory of Dynamic Programming. IX. Variational Analysis, Analytic Continuation, and Imbedding of Operation . . . . . *By Richard Bellman and Sherman Lehman* 905
On the Semiradical of a Semiring . . . . . *By Samuel Bourne and Hans Zassenhaus* 907
The Strong Minkowski-Farkas-Weyl Theorem for Vector Spaces over Ordered Fields . . . . . *By A. Charnes and W. W. Cooper* 914
Supplement to a Paper Entitled "A Von Sterneck Arithmetical Function and Restricted Partitions with Respect to a Modulus" . . . . . *By C. A. Nicol and H. S. Vandiver* 917

GENERATING FUNCTIONS AND THE SEMIGROUP THEORY OF BRANCHING MARKOV PROCESSES . . . By A. T. Bharucha-Reid and Herman Rubin 1057
THE WORD PROBLEM . . . By William W. Boone 1061
NON-ADDITIVE FUNCTIONS, THEIR DERIVED FUNCTORS, AND THE SUSPENSION HOMOMORPHISM . . . By Albrecht Dold and Dieter Puppe 1065
DIFFERENTIABLE MAPPINGS IN THE SCHOENFLIES PROBLEM . . . By Marston Morse 1068
COHOMOLOGY THEORY OF VARIETIES OVER RINGS . By Wei-Liang Chow and Jun-ichi Igusa 1244
ON THE FOUNDATIONS OF THE THEORY OF DISTRIBUTIONS . . . By I. S. Gál 1248
ON THE SPECTRAL THEORY OF SINGULAR INTEGRAL OPERATORS . . By Walter Koppelman 1252

ASTRONOMY

ON FORCE-FREE MAGNETIC FIELDS . . . By S. Chandrasekhar and L. Woltjer 285
A THEOREM ON FORCE-FREE MAGNETIC FIELDS . . . By L. Woltjer 489
ON HYDROMAGNETIC EQUILIBRIUM . . . By L. Woltjer 833
ON THE EQUILIBRIUM CONFIGURATION OF AN INCOMPRESSIBLE FLUID WITH AXISYMMETRIC MOTIONS AND MAGNETIC FIELDS . . . By S. Chandrasekhar 842

PHYSICS

SPIN, STATISTICS, AND THE TCP THEOREM . . . By Julian Schwinger 223
INVARIANT IMBEDDING, WAVE PROPAGATION, AND THE WKB APPROXIMATION . . . By Richard Bellman and Robert Kalaba 317
INVARIANT IMBEDDING RELATION FOR THE PRINCIPLES OF INVARIANCE . . . By Rudolph W. Preisendorfer 320
FUNCTIONAL RELATIONS FOR THE R AND T OPERATORS ON PLANE-PARALLEL MEDIA . . . By Rudolph W. Preisendorfer 323
TIME-DEPENDENT PRINCIPLES OF INVARIANCE . . . By Rudolph W. Preisendorfer 328
l-TYPE DOUBLING IN NH_3 . . . By John S. Garing and Harald H. Nielsen 467
ON THE NATURE OF FREE RADICALS DETECTED BY PARAMAGNETIC RESONANCE IN BIOLOGICAL SUBSTANCES . . . By Ichiro Miyagawa, Walter Gordy, Norimitsu Watabe, and Karl M. Wilbur 613
ADDENDUM TO SPIN, STATISTICS, AND THE TCP THEOREM . . . By Julian Schwinger 617
RADIOACTIVE FALLOUT . . . By W. F. Libby 800
ON THE DIFFUSE REFLECTION OF A PENCIL OF RADIATION BY A PLANE-PARALLEL ATMOSPHERE . . . By S. Chandrasekhar 933
REMARKS ON THE LONG-WAVE LIMITS OF PHOTOSYNTHESIS AND CHLOROPHYLL FLUORESCENCE . . . By James Franck 941
CRYSTAL STRUCTURE AND INFORMATION THEORY . . . By Dan McLachlan, Jr. 948
ON THE EUCLIDEAN STRUCTURE OF RELATIVISTIC FIELD THEORY . . . By Julian Schwinger 956
THE THERMAL CONDUCTIVITY OF CARBON DIOXIDE IN THE REGION OF THE CRITICAL POINT . . . By Leslie A. Guildner 1149

CHEMISTRY

CONDUCTANCE OF TETRABURYLAMMONIUM TETRAPHENYLBORIDE . . . By Raymond M. Fuoss, Joan B. Berkowitz, Ernest Hirsch, and Sergio Petrucci 27
A THEORY OF OPTIMUM CONDITIONS FOR PHASE TRANSITIONS . . . By Henry Eyring, F. W. Cagle, Jr., and Carl J. Christensen 120
THE INACTIVATION OF GROWTH-PROMOTING DISLOCATIONS WITH TEMPERATURE, PRESSURE, AND POISONS . . . By James E. Manson, F. W. Cagle, Jr., and Henry Eyring 156
EARLY UNSTABLE CO_2 FIXATION PRODUCTS IN PHOTOSYNTHESIS . . . By Helmut Metzner, Barbara Metzner, and Melvin Calvin 205
THE NATURE OF BOND ORBITALS AND THE ORIGIN OF POTENTIAL BARRIERS TO INTERNAL ROTATION IN MOLECULES . . . By Linus Pauling 211
PRINCIPLE OF MINIMUM BENDING OF LOCALIZED AND DELOCALIZED ORBITALS: ETHANE BARRIER AND RELATED EFFECTS . . . By Henry Eyring, George H. Stewart, and Richard P. Smith 259

THE PATH OF CARBON IN PHOTOSYNTHESIS. XXII. THE IDENTIFICATION OF CARBOXYKETOPENTITOL DIPHOSPHATES AS PRODUCTS OF PHOTOSYNTHESIS . . . . . *By V. Moses and M. Calvin* 260
SPONTANEOUS NUCLEATION IN SUPER-SATURATED VAPORS . . . . . *By Worth H. Rodebush and John V. Clarke, Jr.* 536
SIGNIFICANT STRUCTURES IN THE LIQUID STATE . . . . . *By Henry Eyring, Taikyue Ree, and Nishio Hirai* 683
THE MODE OF PROTONATION OF AMIDES . . . . . *By Gideon Fraenkel and Carl Niemann* 688
A PSEUDOVECTOR NUCLEAR HYPERFINE INTERACTION . . . . *By Harden M. McConnell* 766
PARAMAGNETIC RESONANCE STUDY OF IRRADIATION DAMAGE IN CRYSTALLINE CARBOHYDRATES . . *By Dudley Williams, J. E. Geusic, M. L. Wolfrom, and Leo J. McCabe* 1127
THE VISCOSITY OF HIGH POLYMERS—THE RANDOM WALK OF A GROUP OF CONNECTED SEGMENTS . . . . . *By Henry Eyring, Taikyue Ree, and Nishio Hirai* 1213

BOTANY

SYMPOSIUM ON PLANT TUMORS: INTRODUCTION . . . . . *By A. J. Riker* 338
A TREE TUMOR OF UNKNOWN ORIGIN . . . . . *By Philip R. White* 339
A PHYSIOLOGICAL BASIS FOR AUTONOMOUS GROWTH OF THE CROWN-GALL TUMOR CELL . . . . . *By Armin C. Braun* 344
ACTIVATION OF METABOLIC SYSTEMS DURING TUMOR-CELL FORMATION . . . . . *By Richard W. Klein* 350
STIMULATION OR INHIBITION OF VIRUS INFECTED AND INSECT-GALL TISSUES AND SINGLE-CELL CLONES . . . . . *By A. C. Hildebrandt* 354
WOUND-TUMOR . . . . . *By L. M. Black* 363
CONCLUSIONS . . . . . *By A. J. Riker* 367
WALL THICKENING IN SIEVE ELEMENTS . . . *By Katherine Esau and Vernon I. Cheadle* 546
THE FUNCTION OF EXTRACELLULAR ENZYMES OF DUTCH ELM DISEASE PATHOGEN . . . . . *By Akhtar Husain and A. E. Dimond* 594
SUCROSE AND GROWTH OF EXCISED ROOTS OF AN INBRED *Zea Mays* . . . . . *By William J. Robbins* 1210

ZOÖLOGY

DIFFERENCE IN ELECTRIC POTENTIAL ACROSS THE PLACENTA OF GOATS . . . . . *By G. Meschia, A. S. Wolkoff, and D. H. Barron* 483
ACTION OF NEUROSECRETION UPON CENTRAL NERVOUS SYSTEM IN VITRO AND UPON BEHAVIOR . . . . . *By S. Ozbas and E. S. Hodgson* 825
TETRAPOD EXTINCTIONS AT THE END OF THE TRIASSIC PERIOD . . *By Edwin H. Colbert* 973
DIRECT EVIDENCE FOR THE TRANSFER OF MATERIALS FROM SYMBIOTIC ALGAE TO THE TISSUES OF A COELENTERATE . . . . . *By Leonard Muscatine and Cadet Hand* 1259
THYMUS RIBONUCLEIC ACID AND EMBRYONIC DIFFERENTIATION . . . . . *By M. C. Niu* 1264
CARTILAGE FORMATION IN SOMITE GRAFTS OF CHICK BLASTODERM . . . . . *By Tamikazu Seno and Ilhan Büyüközer* 1274

GENETICS

CHROMOSOMAL POLYMORPHISM IN CARIBBEAN ISLAND POPULATIONS OF *Drosophila Willistoni* . . . . . *By J. Ives Townsend* 38
INDUCTION OF SPECIFIC MUTATIONS WITH 5-BROMOURACIL . . . . . *By Seymour Benzer and Ernst Freese* 112
A PRELIMINARY INVESTIGATION OF PSEUDOALLELISM AT THE NOTCH LOCUS OF *Drosophila Melanogaster* . . . . . *By W. J. Welshons* 254
ABERRANT SEGREGATION AND PSEUDOALLELISM AT THE GARNET LOCUS IN *Drosophila Melanogaster* . . . . . *By Arthur Chovnick* 333
EVIDENCE FROM TETRAD ANALYSIS FOR BOTH NORMAL AND ABERRANT RECOMBINATION BETWEEN ALLELIC MUTANTS IN *Neurospora Crassa* . . . . . *By Mary E. Case and Norman H. Giles* 378
MODIFICATION OF ULTRAVIOLET-INDUCED MUTATION FREQUENCY AND SURVIVAL IN BACTERIA BY POST-IRRADIATION TREATMENT . . *By C. O. Doudney and F. L. Haas* 390

THE MECHANISM OF CHEMICAL MUTAGENESIS. I. KINETIC STUDIES ON THE ACTION OF TRIETHYLENE MELAMINE (TEM) AND AZASERINE . . . . . *By V. J. Iyer and Waclaw Szybalski* 446

INFLUENCE OF THE NUCLEOLUS ON MITOSIS AS REVEALED BY ULTRAVIOLET MICROBEAM IRRADIATION . . . . . *By Mary Esther Gaulden and Robert P. Perry* 553

THE IMMUNE STATUS OF LONG-TERM SURVIVORS OF LETHALLY X-IRRADIATED MICE PROTECTED WITH ISOLOGOUS, HOMOLOGOUS, OR HETEROLOGOUS BONE MARROW . . . . . *By N. Gengozian, T. Makinodan, C. C. Congdon, and R. D. Owen* 560

GENETIC STUDIES OF IRRADIATED NATURAL POPULATIONS OF *Drosophila*. II. 1957 TESTS . . . . . *By Wilson S. Stone and Florence D. Wilson* 565

INTERRACIAL HYBRIDIZATION AND BREAKDOWN OF COADAPTED GENE COMPLEXES IN *Drosophila Willistoni* . . . . . *By Theodosius Dobzhansky and Olga Pavlovsky* 622

THE REPLICATION OF DNA IN *Escherichia Coli* . . . . . *By Matthew Meselson and Franklin W. Stahl* 671

OCCURRENCE OF CHROMOSOMAL ABERRATIONS IN PRE-SPERMATOCYTIC CELLS OF IRRADIATED MALE MICE . . . . . *By A. B. Griffen* 691

EPIGENETIC CONTROL SYSTEMS . . . . . *By D. L. Nanney* 712

ON THE NATURE OF THE GARNET LOCUS IN *Drosophila Melanogaster* . . . . . *By W. M. Hexter* 768

ACTION OF RADIATION ON MAMMALIAN CELLS III. RELATIONSHIP BETWEEN REPRODUCTIVE DEATH AND INDUCTION OF CHROMOSOME ANOMALIES BY X-IRRADIATION OF EUPLOID HUMAN CELLS IN VITRO . . . . . *By Theodore T. Puck* 772

THE STRUCTURE OF THE INCOMPATIBILITY FACTORS IN SCHIZOPHYLLIUM COMMUNE . . . . . *By John R. Raper, Margery G. Baxter, and Richard B. Middleton* 889

RADIATION-INDUCED MUTATION RATES IN FEMALE MICE . . . . . *By W. L. Russell, Liane Brauch Russell, Josephine S. Gower, and Savanna C. Maddux* 901

THE PRESENCE OF A_2 ERYTHROCYTES IN A_1 BLOOD . . . . . *By K. C. Atwood* 1054

INCREASE IN FITNESS IN EXPERIMENTAL POPULATIONS RESULTING FROM HETEROSIS . . . . . *By Hampton L. Carson* 1136

BIOLOGICAL DAMAGE IN DEVELOPING GERM CELLS OF *Drosophila Virilis* IN OXYGEN AND NITROGEN WITH 14-MEV NEUTRONS . . . . . *By Mary L. Alexander* 1217

THE SOMATIC CHROMOSOMES OF MAN . . . . . *By J. H. Tjio and T. T. Puck* 1229

COMPLEMENTATION AT THE AD-4 LOCUS IN *Neurospora Crassa* . . . . . *By Dow O. Woodward, C. W. H. Partridge, and Norman H. Giles* 1237

PHYSIOLOGY

ON THE MAGNETIC ASYMMETRY OF MUSCLE FIBERS . . . . . *By William Arnold, Richard Steele, and Helmut Mueller* 1

MODIFICATION OF CELL-MIGRATION PATTERNS IN THE EARLY CHICK EMBRYO . . . . . *By Robert L. DeHaan* 32

MONTHLY CYCLES IN AN ORGANISM IN CONSTANT CONDITIONS DURING 1956 AND 1957 . . . . . *By Frank A. Brown, Jr., M. F. Bennett, and H. M. Webb* 290

CELLULAR PROLIFERATION IN THE MOUSE AS REVEALED BY AUTORADIOGRAPHY WITH TRITIATED THYMIDINE . . . . . *By W. L. Hughes, V. P. Bond, G. Brecher, E. P. Cronkite, R. B. Painter, H. Quastler, and F. G. Sherman* 476

THE RELATION BETWEEN LONGEVITY AND OBESITY IN HUMAN BEINGS . *By Linus Pauling* 619

VITAMIN A DEFICIENCY AND NIGHT BLINDNESS . . *By John E. Dowling and George Wald* 648

A MECHANISM OF CELLULAR THERMOGENESIS IN COLD-ADAPTATION . . . . . *By Robert E. Smith and Alan S. Fairhurst* 705

THE HUMAN ELECTROHYSTEROGRAM: WAVE FORMS AND IMPLICATIONS . . *By S. D. Larks* 820

ON THE SIGNIFICANCE OF TRANSIENTS IN DAILY RHYTHMS . . . . . *By Colin Pittendrigh, Victor Bruce, and Peter Kaus* 965

THE APPLICATION OF THE FICK PRINCIPLE TO THE MEASUREMENT OF PULMONARY BLOOD FLOW . . . . . *By H. W. Fritts, Jr., and A. Cournand* 1079

PATHOLOGY AND MICROBIOLOGY

SOME MECHANISMS OF CONSCIOUSNESS DISCOVERED DURING ELECTRICAL STIMULATION OF THE BRAIN . . . *By Wilder Penfield* 51
INACTIVATION OF HUMAN ERYTHROCYTE AGGLUTINOGENS M AND N BY INFLUENZA VIRUSES AND RECEPTOR-DESTROYING ENZYME . . *By Georg F. Springer and Norma J. Ansell* 182
CONCERNING THE ORIGIN OF BACTERIAL VIRUSES . . . . . *By John H. Northrop* 229
ACETATE AS A CALCIUM-SPARING FACTOR IN NITROGEN FIXATION BY *Azotobacter Vinelandii* . . . . . *By Raymond G. Esposito and P. W. Wilson* 472
TRANSDUCTION BY BACTERIOPHAGE P1: ABNORMAL PHAGE FUNCTION OF THE TRANSDUCING PARTICLES . . . . . *By Jane N. Adams and S. E. Luria* 590
NOMENCLATURE OF THE ACTINOMYCINS . . . . . *By Selman A. Waksman, Edward Katz, and Leo C. Vining* 602
PRODUCTION, REPRODUCTION, AND REVERSION OF PROTOPLAST-LIKE STRUCTURES IN THE OSMOTIC STRAIN OF *Neurospora Crassa* . . *By Sterling Emerson and Mary R. Emerson* 668
CYTOLOGICAL AND CYTOCHEMICAL CHANGES IN LIVERS OF WHITE MICE FOLLOWING INTRAPERITONEAL INJECTIONS OF DNA PREPARATIONS FROM BREAST CANCERS OF AGOUTI C_3H MICE . *By Cecilie Leuchtenberger, Rudolf Leuchtenberger, and Edwin Uyeki* 700
ON THE NATURE OF THE SELECTIVE EFFECTS OF DEOXYRIBONUCLEIC ACID DIGEST UPON PNEUMOCOCCI OF DIFFERENT VIRULENCE . . *By William Firshein and Werner Braun* 918
EFFECTS OF REDUCED OXYGEN TENSION ON GROWTH RESPONSES OF METHIONINE AND VITAMIN B_{12} STIMULATED MUTANTS OF COLIFORM BACTERIA . *By Lloyd S. Lockingen* 924
TRANSFORMATION OF BIOCHEMICALLY DEFICIENT STRAINS OF *Bacillus Subtilis* BY DEOXYRIBONUCLEATE . . . . . *By John Spizizen* 1072
DISSOCIATION OF RUBIDIUM UPTAKE BY *Neurospora Crassa* INTO ENTRY AND BINDING PHASES . . . . . *By Gabriel Lester and Oscar Hechter* 1141

PSYCHOLOGY

THE SPECTRAL LUMINOSITY CURVES FOR A DICHROMATIC EYE AND A NORMAL EYE IN THE SAME PERSON . . . . . *By C. H. Graham and Yun Hsia* 46
COCHLEAR POTENTIALS IN THE CAT IN RESPONSE TO HIGH-FREQUENCY SOUNDS . . . . . *By Ernest Glen Wever, Jack A. Vernon, Walter E. Rahm, and William F. Strother* 1087
INDIVIDUAL DIFFERENCES IN SUSCEPTIBILITY TO HYPNOSIS . . . . . *By Ernest R. Hilgard, André M. Weitzenhoffer, and Philip Gough* 1255

GEOPHYSICS

CHEMICAL AERONOMY . . . . . *By Joseph Kaplan and Charles A. Barth* 105
SEISMIC SURFACE WAVES AT PALISADES FROM EXPLOSIONS IN NEVADA AND THE MARSHALL ISLANDS . . . . . *By Jack Oliver and Maurice Ewing* 780

BIOCHEMISTRY

MOLECULAR GROWTH REQUIREMENTS OF SINGLE MAMMALIAN CELLS: THE ACTION OF FETUIN IN PROMOTING CELL ATTACHMENT TO GLASS . . . . . *By Harold W. Fisher, Theodore T. Puck, and Gordon Sato* 4
THE MECHANISM OF ACTION OF ALDOLASE AND THE ASYMMETRIC LABELING OF HEXOSE . . . . . *By Irwin A. Rose* 10
ACTIVATION OF HYDROGEN TRANSFER BETWEEN PYRIDINE NUCLEOTIDES BY STEROID HORMONES . . . . . *By Paul Talalay and H. G. Williams-Ashman* 15
SYMPOSIUM ON AMINO ACID ACTIVATION: INTRODUCTION . . . . . *By Fritz Lipmann* 67
INTERMEDIATE REACTIONS IN AMINO ACID INCORPORATION . . . . . *By Paul C. Zamecnik, M. L. Stephenson, and L. I. Hecht* 73
AN ENZYMATIC MECHANISM FOR LINKING AMINO ACIDS TO RNA . . . . . *By P. Berg and E. J. Ofengand* 78
SOME PROBLEMS CONCERNING THE ACTIVATION OF AMINO ACIDS . . . . . *By G. David Novelli* 86

Tracing of the In Vivo Path from Amino Acid to Protein . . . . . By P. D. Boyer and M. P. Stulberg 92
Application of a Theory of Enzyme Specificity to Protein Synthesis . . . . . By D. E. Koshland, Jr. 98
The Visual System of the Honeybee . . . . . By Timothy H. Goldsmith 123
The Luciferin-Luciferase Reaction in a Fish, *Parapriacanthus Beryciformis*, of Newly Discovered Luminescence . . . By Yata Haneda and Frank H. Johnson 127
The Action of Light on Rhodopsin . . . . . By Ruth Hubbard and Allen Kropf 130
On Resonance Transfer of Excitation Energy between Aromatic Amino Acids in Proteins . . . By George Karreman, Richard H. Steele, and Albert Szent-Györgyi 140
The Dependence of Pneumococcal Transformation on the Molecular Weight of Deoxyribose Nucleic Acid . . . . . By Michael Litt, Julius Marmur, Harriett Ephrussi-Taylor, and Paul Doty 144
Observations in Myelin Structure: Incisures and Nodal Regions . . . . . By Mario Luxoro 152
On the Enzymic Activity of Subtilisin-Modified Ribonuclease . . . . . By Frederic M. Richards 162
On the Mechanism of Squalene Biogenesis . . . . . By H. Rilling, T. T. Tchen, and Konrad Bloch 167
The Incorporation of Amino Acids into Ribonucleic Acid . . . . . By Richard S. Schweet, Freeman C. Bovard, Esther Allen, and Edward Glassman 173
Fluorescence Studies of Coenzyme Binding to Dehydrogenases . . . . . By Sidney Shifrin and Nathan O. Kaplan 177
Amino Acid Incorporation in Pigeon Pancreas Fractions . . . . . By Samuel B. Weiss, George Acs, and Fritz Lipmann 189
A Probable Mechanism of Action of Serotonin . . . . . By D. W. Woolley 197
Identification of Blood Characteristics Common to Alcoholic Males . . . . . By Roger J. Williams, Richard B. Pelton, Hertta-Maija Hakkinen, and Lorene L. Rogers 216
Dehydrated Muscle Fibers . . . . . By Helmut Mueller 235
Pteridines in Photosynthesis . . . . By R. C. Fuller, I. C. Anderson, and H. A. Nathan 239
The Enzymatic Activity Associated with the Protein Immunologically Related to Tryptophan Synthetase . . . . . By Charles Yanofsky and Joan Stadler 245
Development of Collagen in the Frog Embryo . . . . . By Mac V. Edds, Jr. 296
Amino Acid Incorporation and the Reversion of its Initial Phase with Cell-Free Tetrahymena Preparations . . . . . By J. Mager and Fritz Lipmann 305
Protein Turnover in Micro-Organisms . . . . . By Ernest Borek, Laura Ponticorvo, and D. Rittenberg 369
The Transfer of Irradiation-Elicited Induction in a Lysogenic Organism . . . . . By Ernest Borek and Ann Ryan 374
The Initiator Cell for Slime Mold Aggregation . . . . . By Herbert L. Ennis and Maurice Sussman 401
The Sonic Fragmentation of Collagen Macromolecules . . . . . By Tomio Nishihara and Paul Doty 411
Interaction Properties of Sonically Fragmented Collagen Macromolecules . . . . . By Alan J. Hodge and Francis O. Schmitt 418
The Solution Properties and Configuration of a Polyampholytic Polypeptide. Copoly-L-Lysine-L-Glutamic Acid . . . By P. Doty, K. Imahori, and E. Klemperer 424
The Properties of Sonic Fragments of Deoxyribose Nucleic Acid . . . . . By Paul Doty, Barbara Bunce McGill, and Stuart A. Rice 432
Labeling of Proteins by Isotopic Amino Acid Derivatives . . . . . By Christine Zioudrou, Setsuro Fujii, and Joseph S. Fruton 439
The Effects of Hydroxylamine on the $C_{14}O_2$ Fixation Pattern during Photosynthesis . . . . . By J. A. Bassham, M. Kirk, and M. Calvin 491
Protein Biosynthesis by a Cell-Free Bacterial System . . . . . By Mirko Beljanski and Severo Ochoa 494

VITAMIN K, AN ESSENTIAL FACTOR FOR THE PHOTOCHEMICAL ACTIVITY OF ISOLATED CHLOROPLASTS . . . *By Norman I. Bishop* 501

IONIZATION OF INDIVIDUAL GROUPS IN DIBASIC ACIDS, WITH APPLICATION TO THE AMINO AND HYDROXYL GROUPS OF TYROSINE . . . *By John T. Edsall, R. Bruce Martin, and Barbara K. Hollingworth* 505

PTERIDINES IN PHOTOSYNTHESIS—AN ARTIFACT OF PAPER CHROMATOGRAPHY . . . *By R. C. Fuller, I. C. Anderson, and H. A. Nathan* 518

ON THE EMISSION OF TRYPTOPHAN . . . *By Irvin Isenberg and Albert Szent-Györgyi* 519

ON DISSOCIATION AND RECOMBINATION OF HUMAN ADULT HEMOGLOBINS A, S, AND C . . . *By Harvey A. Itano and S. J. Singer* 522

EFFECT OF AG[illegible] ON THE ENZYME ACTIVITY IN ERYTHROCYTES . . . *By Paul A. Marks, Anne B. Johnson, and Erich Hirschberg* 529

STUDIES ON THE EXCITED STATES OF PROTEINS . . . *By Richard H. Steele and Albert Szent-Györgyi* 540

ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID. III. THE INCORPORATION OF PYRIMIDINE AND PURINE ANALOGUES INTO DEOXYRIBONUCLEIC ACID . . . *By Maurice J. Bessman, I. R. Lehman, Julius Adler, Steven B. Zimmerman, E. S. Simms, and Arthur Kornberg* 633

ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID. IV. LINKAGE OF SINGLE DEOXYNUCLEOTIDES TO THE DEOXYNUCLEOSIDE ENDS OF DEOXYRIBONUCLEIC ACID . . . *By Julius Adler, I. R. Lehman, Maurice J. Bessman, E. S. Simms, and Arthur Kornberg* 641

MOLECULAR INTERACTION OF ISOALLOXAZINE DERIVATIVES . . . *By Henry A. Harbury and Kathryn A. Foley* 662

ENZYMATIC FORMATION OF ADENYL TRYPTOPHAN: ISOLATION AND IDENTIFICATION . . . *By Henry S. Kingdon, Leslie T. Webster, Jr., and Earl W. Davie* 757

THIOLATION OF PROTEINS . . . *By Reinhold Benesch and Ruth E. Benesch* 848

THE INTERPRETATION OF BACTERIAL TRANSFORMATION DATA . . . *By Liebe F. Cavalieri and Barbara H. Rosenberg* 853

FREE RADICAL FORMATION IN RIBOFLAVIN COMPLEXES . . . *By Irvin Isenberg and Albert Szent-Györgyi* 857

ON A COENZYMATIC FUNCTION OF ESTRADIOL-17-β . . . *By Paul Talalay, Barbara Hurlock, and H. G. Williams-Ashman* 862

ISOLATION OF ADENOSINE AMINO ACID ESTERS FROM A RIBONUCLEASE DIGEST OF SOLUBLE, LIVER RIBONUCLEIC ACID . . . *By Hans Georg Zachau, George Acs, and Fritz Lipmann* 885

SOME EFFECTS OF SUBSTITUTING THE DEOXYRIBONUCLEIC ACID OF ISOLATED NUCLEI WITH OTHER POLYELECTROLYTES . . . *By V. G. Allfrey and A. E. Mirsky* 981

A NEW WAY FOR THE SYNTHESIS OF 3-AMINO SUGARS . . . *By Hans Helmut Baer and Hermann O. L. Fischer* 991

THE ENZYMATIC SYNTHESIS OF FATTY ACIDS BY ALDOL CONDENSATION . . . *By Roscoe O. Brady* 993

PHOSPHORYLATED INTERMEDIATES IN THE SYNTHESIS OF SQUALENE . . . *By S. Chaykin, J. Law, A. H. Phillips, T. T. Tchen, and K. Bloch* 998

THE MODE OF ACTION OF 5-FLUOROURACIL AND ITS DERIVATIVES . . . *By Seymour S. Cohen, Joel G. Flaks, Hazel D. Barner, Marilyn R. Loeb, and Janet Lichtenstein* 1004

CREVICE STRUCTURES IN HEMOPROTEIN REACTIONS *By Philip George and R. L. J. Lyster* 1013

THE SYNTHESIS OF HEMOGLOBIN IN A CELL-FREE SYSTEM . . . *By Richard Schweet, Hildegarde Lamfrom, and Esther Allen* 1029

DELAYED LIGHT EMISSION IN GREEN PLANT MATERIALS: TEMPERATURE-DEPENDENCE AND QUANTUM YIELD . . . *By G. Tollin, E. Fujimori, and M. Calvin* 1035

THE BIOSYNTHESIS OF ISOLEUCINE AND VALINE IN *Neurospora Crassa* . . . *By R. P. Wagner, A. N. Radhakrishnan, and E. E. Snell* 1047

A COENZYME CONTAINING PSEUDOVITAMIN B_{12} . . . *By H. A. Barker, H. Weissbach, and R. D. Smyth* 1093

ON THE REGULATION OF FATTY ACID BIOSYNTHESIS BY LIPOGENIN . . . *By G. N. Catravas and H. S. Anker* 1097

Electron-Spin Resonance Studies of Free-Radical Intermediates in Oxidation-Reduction Enzyme Systems . . . . . *By Barry Commoner, Barbara B. Lippincott, and Janet V. Passonneau* 1099

Light-Induced Free Radicals in FMN and Flavoprotein Enzymes . . . . . *By Barry Commoner and Barbara B. Lippincott* 1110

Linear Analysis of Tobacco Mosaic Virus . . . . . *By Barry Commoner, Georgia B. Shearer, and Clarice Strode* 1117

A Synthesis of Ribothymidine-5′-Pyrophosphate and Its Conversion to Polyribothymidylic Acid *By Beverly E. Griffin, Sir Alexander Todd, and Alexander Rich* 1123

Protein Biosynthesis by a Cell-Free Bacterial System. II. Further Studies on the Amino Acid Incorporation Enzyme . *By Mirko Beljanski and Severo Ochoa* 1157

On the Separation of the Tryptophan Synthetase of *Escherichia Coli* into Two Protein Components . . . . . *By Irving P. Crawford and Charles Yanofsky* 1161

Dormancy of Bacterial Endospores: Regulation of Electron Transport by Dipicolinic Acid . . . . . *By Harlyn Halvorson, Roy Doi, and Brooks Church* 1171

Observations on the Function of Pyridoxal-5-Phosphate in Phosphorylase . . . . . *By Barbara Illingworth, Hendrik S. Jansz, David H. Brown, and Carl F. Cori* 1180

Enzymatic Synthesis of Deoxyribonucleic Acid. V. Chemical Composition of Enzymatically Synthesized Deoxyribonucleic Acid . . *By I. R. Lehman, Steven B. Zimmerman, Julius Adler, Maurice J. Bessman, E. S. Simms, and Arthur Kornberg* 1191

Electron-Donor and -Acceptor Properties of Biologically Important Purines, Pyrimidines, Pteridines, Flavins, and Aromatic Amino Acids . . . . . *By Bernard Pullman and Alberte Pullman* 1197

Serotonin Receptors. I. Extraction and Assay of a Substance Which Renders Serotonin Fat-Soluble . . . . . *By D. W. Woolley* 1202

ERRATA

The Formation of Antibodies in Man after Injection of Pneumoccal Polysaccharides . . . . . *By Michael Heidelberger* 485

Stimulation or Inhibition of Virus Infected and Insect-Gall Tissues and Single-Cell Clones . . . . . *By A. C. Hildebrandt* 718

Nomenclature of the Actinomycins . . . . . *By Selman A. Waksman, Edward Katz, and Leo C. Vining* 718

Dynamic Programming, Successive Approximations, and Monotone Convergence . . . . . *By Richard Bellman* 978

Invariant Imbedding, Wave Propagation, and the WKB Approximation . . . . . *By Richard Bellman and Robert Kalaba* 978

Differentiable Mappings in the Schoenflies Problem . . . . *By Marston Morse* 1285

INFORMATION TO CONTRIBUTORS

THE PROCEEDINGS is the official organ of the NATIONAL ACADEMY OF SCIENCES and of the NATIONAL RESEARCH COUNCIL for the publication of brief accounts of important current researches of members of the ACADEMY and of the COUNCIL and of other American investigators. THE PROCEEDINGS will aim especially to secure prompt publication of original announcements of discoveries and wide circulation of the results of American research among investigators in other countries and in all branches of science.

ARTICLES should be brief. The viewpoint should be comprehensive in giving the relation of the paper to previous publications of the author or of other and in exhibiting, where practicable, the significance of the work for other branches of science. Elaborate technical details of the work and long tables of data should be avoided, but authors should be precise in making clear the new results and should give some record of the methods and data upon which they are based.

MANUSCRIPTS should be prepared with a current number of THE PROCEEDINGS as a model in matters of form, and should be typewritten in duplicate with double spacing, the author retaining one copy. Illustrations should be confined to text figures of simple character, though more elaborate illustration may be allowed in special instances to authors willing to pay for their preparation and insertion. Particular attention should be given to arranging tabular matter in a simple and concise manner.

MANUSCRIPTS will be accepted only from members of the Academy who will assume responsibility for the propriety and scientific standards of the paper and for any printing costs in excess of those allowed them by the Academy. Manuscripts should be addressed to THE PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, 2101 Constitution Ave., Washington 25, D. C.

GALLEY PROOF will be sent, with the understanding that charges for author's corrections shall be billed to him. Author should therefore make final revisions on the typewritten manuscripts. Original artwork or figures cannot be returned unless a request is made when the manuscript is submitted. Page proofs will not be sent.

REPRINTS should be ordered when proof is returned, on the reprint order form which will be inclosed with the proof. They will be furnished at cost, approximately as follows:

SCHEDULE OF REPRINT RATES (POSTAGE NOT INCLUDED)

Quantity	up to 4 pp.	up to 8 pp.	up to 12 pp.	Standard Covers
50	$ 7.95	$15.10	$21.50	$ 6.95
100	9.05	17.45	25.85	8.50
150	10.15	19.65	29.20	10.00
add'l 100's	2.25	4.45	6.65	3.05

CONTENTS

Page

BIOCHEMISTRY.—PROTEIN BIOSYNTHESIS BY A CELL-FREE BACTERIAL SYSTEM. II. FURTHER STUDIES ON THE AMINO ACID INCORPORATION ENZYME . . . . . *By Mirko Beljanski and Severo Ochoa* 1157

BIOCHEMISTRY.—ON THE SEPARATION OF THE TRYPTOPHAN SYNTHETASE OF ESCHERICHIA COLI INTO TWO PROTEIN COMPONENTS . *By Irving P. Crawford and Charles Yanofsky* 1161

BIOCHEMISTRY.—DORMANCY OF BACTERIAL ENDOSPORES: REGULATION OF ELECTRON TRANSPORT BY DIPICOLINIC ACID . *By Harlyn Halvorson, Roy Doi, and Brooks Church* 1171

BIOCHEMISTRY.—OBSERVATIONS ON THE FUNCTION OF PYRIDOXAL-5-PHOSPHATE IN PHOSPHORYLASE . . . . . *By Barbara Illingworth, Hendrik S. Janez, David H. Brown, and Carl F. Cori* 1180

BIOCHEMISTRY.—ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID. V. CHEMICAL COMPOSITION OF THE ENZYMATICALLY SYNTHESIZED DEOXYRIBONUCLEIC ACID . . . . . *By I. R. Lehman, Steven B. Zimmerman, Julius Adler, Maurice J. Bessman, E. S. Simms, and Arthur Kornberg* 1191

BIOCHEMISTRY.—ELECTRON-DONOR AND -ACCEPTOR PROPERTIES OF BIOLOGICALLY IMPORTANT PURINES, PYRIMIDINES, PTERIDINES, FLAVINS, AND AROMATIC AMINO ACIDS . . . . . *By Bernard Pullman and Alberte Pullman* 1197

BIOCHEMISTRY.—SEROTONIN RECEPTORS. I. EXTRACTION AND ASSAY OF A SUBSTANCE WHICH RENDERS SEROTONIN FAT-SOLUBLE . . . . . *By D. W. Woolley* 1202

BOTANY.—SUCROSE AND GROWTH OF EXCISED ROOTS OF AN INBRED ZEA MAYS . . . . . *By William J. Robbins* 1210

CHEMISTRY.—THE VISCOSITY OF HIGH POLYMERS—THE RANDOM WALK OF A GROUP OF CONNECTED SEGMENTS . . . . . *By Henry Eyring, Taikyue Ree, and Nishio Hirai* 1213

GENETICS.—BIOLOGICAL DAMAGE IN DEVELOPING GERM CELLS OF DROSOPHILA VIRILIS IN OXYGEN AND NITROGEN WITH 14-MEV NEUTRONS . . . . . *By Mary L. Alexander* 1217

GENETICS.—THE SOMATIC CHROMOSOMES OF MAN . . *By J. H. Tjio and T. T. Puck* 1229

GENETICS.—COMPLEMENTATION AT THE AD-4 LOCUS IN NEUROSPORA CRASSA . . . . . *By Dow O. Woodward, C. W. H. Partridge, and Norman H. Giles* 1237

MATHEMATICS.—COHOMOLOGY THEORY OF VARIETIES OVER RINGS . . . . . *By Wei-Liang Chow and Jun-Ichi Igusa* 1244

MATHEMATICS.—ON THE FOUNDATIONS OF THE THEORY OF DISTRIBUTIONS *By I. S. Gál* 1248

MATHEMATICS.—ON THE SPECTRAL THEORY OF SINGULAR INTEGRAL OPERATORS . . . . . *By Walter Koppelman* 1252

PSYCHOLOGY.—INDIVIDUAL DIFFERENCES IN SUSCEPTIBILITY TO HYPNOSIS . . . . . *By Ernest R. Hilgard, André M. Weitzenhoffer, and Philip Gough* 1255

ZOÖLOGY.—DIRECT EVIDENCE FOR THE TRANSFER OF MATERIALS FROM SYMBIOTIC ALGAE TO THE TISSUES OF A COELENTERATE . . *By Leonard Muscatine and Cadet Hand* 1259

ZOÖLOGY.—THYMUS RIBONUCLEIC ACID AND EMBRYONIC DIFFERENTIATION . . . . . *By M. C. Niu* 1264

ZOÖLOGY.—CARTILAGE FORMATION IN SOMITE GRAFTS OF CHICK BLASTODERM . . . . . *By Tamikazu Seno and Ilhan Büyüközer* 1274